Mass Spectrometry-Based Lipidomics Indicates that Consumption of Fatty Fish Alters the lipid Species Profile of Human LDL

Master´s thesis

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Tiivistelmä – Referat – Abstract

Lipoproteins are biochemical carriers of the insoluble lipids. They are complexes combining lipids and proteins for the transport of lipids. Amongst the type of lipoproteins are low-density lipoproteins (LDL) which are prevalent in various diseases such as obesity, diabetes, atherosclerosis, and other cardiovascular diseases (CVD). Omega-3 fatty acids are polyunsaturated fatty acids (PUFA) that are essential components of lipid metabolism and play a significant role in the human diet. Omega-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are derived from fish and are necessary for proper cardiovascular functioning. Because the human body is unable to produce enough quantities of some omega-3, diet is an important source for its availability. When a diet is rich in saturated fats, the above-mentioned diseases transpire. This study investigated how consumption of two fish diets, Lean fish and Fatty fish, influence the lipid species of human LDL particles. The lipid species analysed in this study are phospholipids such as phosphatidylcholine (PC), sphingomyelin (SM), and lysophosphatidylcholine (LPC), and cholesteryl esters (CE), and triacylglycerols (TAG). A total of 42 volunteers with a history of impaired fasting glucose had randomly been divided into two groups: fatty fish (4 fish meals/week) and lean fish (4 fish meals/week) for 12 weeks. Blood samples had been collected from the volunteers before and after consumption of the fish meals and LDL particles had been isolated from the blood samples by ultracentrifugation. In this study, the lipids were extracted by Folch method, and the extracted lipids were analysed using Triple quadrupole mass spectrometry. The lipid class profile did not change due to the two fish type diets. However, the consumption of fatty fish diet increased the levels of lipid species of PC, LPC, and CE containing EPA and DHA acyl chains, while decreasing levels of several TAG species. Lean fish induced minor changes in the lipid composition of LDL particles. Based on these results, fatty fish diet alters the plasma LDL lipidome profile with changes induced to both the surface and the core composition of the LDL particles in a positive way regarding cardiovascular health.

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List of abbreviations

ACAT Acyl-coenzyme A: cholesterol acyltransferase

Acetyl-CoA Acetyl-coenzyme A

ALA alpha-linolenic acid

AGPAT 1 acylglycerol-3-phosphate acyltransferase

AHA American Heart Association

APCI Atmospheric-pressure chemical ionisation

APPI Atmospheric-pressure photoionization

Apo Apolipoprotein

ATP Adenosine triphosphate

CDP-DAG Cytidine diphosphate diacylglycerol

CE Cholesteryl esters

C: M Chloroform: Methanol

CMP Cytidine monophosphate

CTP Cytidine triphosphate

CVD Cardiovascular diseases

DHA Docosahexaenoic acid

ECT CTP: phosphoethanolamine cytidylyltransferase

EK Ethanolamine kinase

EPA Eicosapentaenoic acid

ER Endoplasmic reticulum

ESC European Society of Cardiology

ESI Electrospray ionisation

FF Fatty fish

GPAT glycerol-3-phosphate acyltransferase

HDL High-density lipoprotein

HMG CoA beta-hydroxy-beta-methylglutaryl CoA

hrSMase Human recombinant secretory sphingomyelinase

IDL Intermediate density lipoprotein

IL Interleukin

LCAT Lecithin: cholesterol acyltransferase enzyme

LC-MS Liquid Chromatography-Mass spectrometry

LDL Low-density lipoprotein

LF Lean fish

LPA Lysophosphatidic acid

LPAT Lysophosphatidyl acyltransferase

LpL lipoprotein lipase

LRP LDL receptor-related protein

Malonyl CoA Malonyl coenzyme A

MCP-1 Monocyte chemoattractant protein-1

m/z mass/charge

Mn^{2+} Manganese ion

NADPH Nicotinamide adenine dinucleotide phosphate

NMR Nuclear Magnetic Resonance

OGTT Oral glucose tolerance test

PA Phosphatidic acid

PC Phosphatidylcholine

PCSK9 Proprotein convertase subtilisin/kexin type 9

PE Phosphatidylethanolamine

PG Phosphatidylglycerol

PGP Phosphatidylglycerolphosphate

PI Phosphatidylinositol

PL Phospholipid

$PPARg2^{-/-}$ Peroxisome proliferator-activated receptor gamma 2

PS Phosphatidylserine

PUFA Polyunsaturated fatty acid

Q Quadrupole

SM Sphingomyelin

SRA Scavenger receptor A

SREBP Sterol regulatory element-binding protein

TAG Triacylglycerol

VCAM-1 Vascular cell adhesion molecule-1

VLDL Very low-density lipoprotein

1. Introduction

n-3 polyunsaturated fatty acids (PUFAs) are dietary fats that have anti-inflammatory properties associated with a healthy lifestyle (Swanson, Block, and Mousa 2012). A normal human body is unable to make enough amounts of these fatty acids required for lipid metabolism. Inefficient lipid metabolism creates an imbalance in fatty acids proportions which can cause a multitude of diseases such as atherosclerosis, diabetes, and other cardiovascular diseases (CVD) (Libby 2002; Wong et al. 2013; Nascimento et al. 2018). Furthermore, atherosclerosis which is an inflammatory disease that occurs due to Low-density lipoproteins (LDL) aided accumulation of cholesterol in arteries, which leads to plaque formation (Libby 2002). LDLs are the carriers of insoluble lipids such as cholesterol and triacylglycerols (TAGs) to the plaque formation site.

Diet is an important source for the availability of dietary fatty acids in the body. A lot of diet studies have been conducted to study the availability and effectivity of dietary fatty acids obtained from plant, and animal source (Schwab et al. 2014; Lesná et al. 2013). Fish is another source of n-3 PUFAs, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA). Both play a role in maintaining the viscosity of cell membranes (Conquer et al. 2000; Swanson, Block, and Mousa 2012) and in foetal development (Dunstan et al. 2007; Swanson, Block, and Mousa 2012). Most fish-based diet studies are cohort studies with fish oil supplements. This study was part of a randomized controlled trial conducted with 41 volunteers for 12 weeks. The volunteers were divided into two groups (fatty fish and lean fish) and were provided with four fish meals per week. This study was important because it allowed us to investigate the effects of fish diets on LDL lipid species and hypothesize the changes caused to the surface and the core lipidome of LDL particles.

1.1 Lipids

Lipids are organic compounds present in all cells, forming a major class of biological molecules that include different types of fats, oils, hormones, and are soluble in nonpolar solvents (Muro, Atilla-Gokcumen, and Eggert 2014; Bader et al. 2016a). The standard classes of lipids are fatty acids, TAG, phospholipids (PL), and sterols (Figure 1). Series of various enzymatic steps help in the *de novo* synthesis of lipids. The synthesis starts with the production of fatty acids, which can convert into neutral lipids (Baenke et al. 2013). These lipids are either stored for use in the later stages or have the possibility of turning into signalling lipids like PL (Baenke et al. 2013; Bader et al. 2016b). Most of the lipids are hydrophobic, while some are amphiphilic. The hydrophobic lipids (non-dissolving in water) contain hydrophobic side chains and polar head groups (Muro, Atilla-Gokcumen, and Eggert 2014). There is heterogeneity in lipids found in cells, and this diversity is contributed by the various arrangement of the side chains and polar head groups (Muro, Atilla-Gokcumen, and Eggert 2014). Recent advances in science have shown that in addition to examining modifications in the head groups, recognising and studying the hydrophobic side chains play an important part in understanding functions of lipids (Qifeng Zhang and Wakelam 2014; Muro, Atilla-Gokcumen, and Eggert 2014). For example, Atilla-Gokcumen and her research team showed that dividing HeLa cells show chemical specificity in the regulation of their lipid content, along with side chains of the lipids.

Amphiphilic lipids are the type that has hydrophilic (dissolving in water) and fat-loving properties and function in combination with proteins or other lipids, creating hurdles in the proper understanding of the functions of lipids. This has not stopped researchers in digging deep; various strategies have been implemented in identifying the lipids. They include nuclear magnetic resonance

(NMR), and mass spectrometry (MS), which help in detecting the lipid species and further understand their functions (Muro, Atilla-Gokcumen, and Eggert 2014). Lipids can also be visualised by using fluorescent tags that work either by tagging the lipid itself or tagging the lipid-binding proteins (Muro, Atilla-Gokcumen, and Eggert 2014). Amongst these methods, the most commonly used one is MS, where recent advances help in identifying the role of lipids using small amounts of the samples based on global or targeted lipid-profile analysis (Muro, Atilla-Gokcumen, and Eggert 2014).

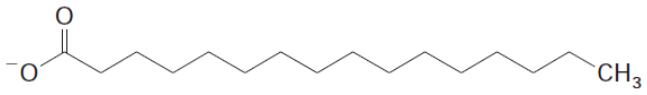
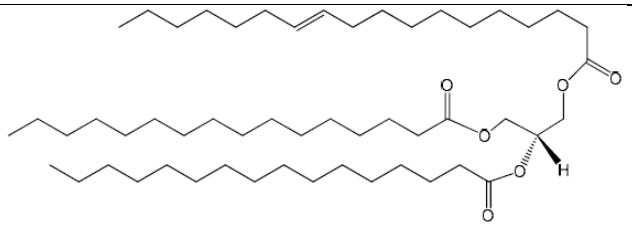
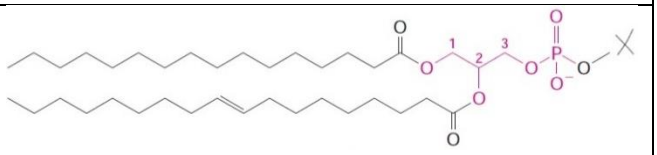
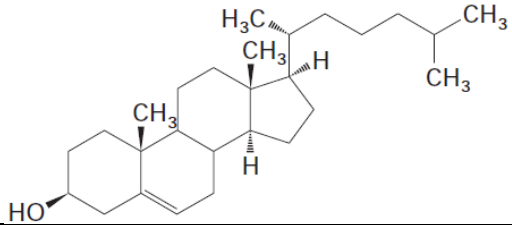
Lipid type	**Function/Role**	**Structure**
Fatty acid (16:0)	Building blocks of lipids	
Triacylglycerols (TAG16:0/16:1/ 18:1)	Storage compound in animal cells	
Phospholipid (X-polar head group)	A major component of cell lipid bilayer	
Sterol (Cholesterol)	Maintain membrane fluidity	
Sphingolipid	Cell recognition, signalling, inflammation	

Figure 1: A modified figure showing common classes of mammalian lipids: Fatty acids (Palmitate (16:0)), Glycerolipids, e.g., TAG (TAG16:0, 16:1, 18:0), PL, Sterol, e.g., cholesterol, and Sphingolipid where X can be charged head group (e.g., ethanolamine, choline, serine)(De Carvalho and Caramujo 2018).

Lipids perform three main functions; firstly they are stored as reservoirs of fatty acids and sterols in an anhydrous state in lipid droplets, which have a core made of neutral lipids such as TAGs, to be used for membrane biogenesis (Van Meer, Voelker, and Feigenson 2008; Melo et al. 2011; Ruggles, Turkish, and Sturley 2013; Bader et al. 2016b). Secondly, lipids present cell membranes the property of cell division and membrane trafficking via budding, fission and other types. These properties are provided via polar lipids that are present in the cell-matrix. The polar lipids have the features of self-association (via hydrophobic moieties) and interacting with the environment (via hydrophilic moieties) (Van Meer, Voelker, and Feigenson 2008). Thirdly, lipids can also participate in signal transduction as first and second messengers, where the degradation of polar lipids provides two separate parts that can be transmitted within the cell through the hydrophobic region and also through the cytosol via the hydrophilic part (Van Meer, Voelker, and Feigenson 2008). Since lipids have vast importance in all cell types, it has been gaining importance amongst researchers and a lot of research is being carried out today.

The majority of the lipids (such as PL, TAG, and sphingolipids) contain both saturated and unsaturated fatty acids, which are also important as building blocks of cellular membranes (De Carvalho and Caramujo 2018). Fatty acid synthesis takes place in the cytoplasm of the cell from acetyl-coenzyme A (acetyl-CoA), Nicotinamide adenosine dinucleotide phosphate (NADPH), biotin and Mn^{2+}, where acetyl-CoA is added to malonyl-coenzyme A (malonyl CoA) in a condensation reaction with carboxylase enzyme acting as the regulatory enzyme. Further elongation of the fatty acids takes place via loading of precursors to thioester derivatives following reduction, dehydration and another reduction reaction steps. β-oxidation is a catabolic process taking place in peroxisomes and mitochondria for the production of energy by converting fatty acids to acetyl-

CoA and NADH through intermediate fatty acyl CoA molecules and further transferred to the citric acid cycle (De Carvalho and Caramujo 2018).

In the *de novo* synthesis of fatty acids, instead of using fatty acids for energy, they are esterified with glycerol and sterol backbones to produce TAGs and sterol esters. These molecules are then stored in lipid droplets (Farese and Walther 2009; Walther and Farese 2012; De Carvalho and Caramujo 2018). Fatty acids can be either saturated or unsaturated. Saturated fatty acids do not have double bonds between carbon atoms, while unsaturated fatty acids contain one or more double bonds. PUFAs have more than one double bond in their acyl chains. In mammals, certain PUFAs are termed as essential dietary fatty acids as they are important for muscle movement, inflammation and are needed from food. Some of the examples of important PUFAs are 20:5 n-3 EPA, and 22:6 n-3 DHA, and 18:3 n-3 α-linolenic acid (ALA) (Monroig, Tocher, and Navarro 2013; De Carvalho and Caramujo 2018). Here n-3 stands for the place of a double bond, which is present three atoms away from the methyl end of the fatty acid. In humans, n-3 PUFAs are essential for various reasons (Swanson, Block, and Mousa 2012). The n-3 PUFAs have various mechanism of actions; they influence metabolites and hormone concentrations, which further affect cell and tissue behaviour. These PUFAs can also affect the oxidation of LDL. Human body is not able to synthesize these PUFAs; therefore, they need to be obtained from the diet either as shorter chain PUFAs such as ALA or as long-chain PUFAs like EPA and DHA (Beermann et al. 2003). Along with n-3 PUFAs, n-6 PUFAs are also important for human health. An imbalance of these fatty acids in the human body can influence inflammation related to multiple diseases, e.g., atherosclerosis, diabetes, and obesity (Weaver et al. 2009; De Carvalho and Caramujo 2018). It is important to have a balance between ingestion of PUFAs

in cells for proper homeostasis and functioning of various tissues (De Carvalho and Caramujo 2018).

Biosynthesis of lipids takes place in many organelles; the main organelle being endoplasmic reticulum (ER). It is the site for the production of structural lipids such as PL and cholesterol as well as lipids performing non-structural roles such as TAG and cholesteryl esters (CE), along with ceramide, which is a precursor for sphingolipids (Bell, Ballas, and Coleman 1981; Van Meer, Voelker, and Feigenson 2008). Lipid synthesis also takes place in the Golgi apparatus and mitochondria. Synthesis of various sphingolipids such as sphingomyelin (SM), glucosylceramide, and lactosylceramide take place in the Golgi (Futerman and Riezman 2005; Van Meer, Voelker, and Feigenson 2008). The synthesized sphingolipids are exported to the plasma membrane, where they are enriched and packed and help resist mechanical stress (Van Meer, Voelker, and Feigenson 2008). Plasma membrane does not take part in the lipid biosynthesis, but various signalling cascade reactions important for synthesis or degradation of its structural lipids take place (Di Paolo and De Camilli 2006; Van Meer, Voelker, and Feigenson 2008). In mitochondria synthesis of lysophosphatidic acid (LPA), a simple glycerophospholipid occurs, and it is used in the formation of TAG, phosphatidylglycerol and phosphatidic acid (PA) (Nagle et al. 2007; Van Meer, Voelker, and Feigenson 2008).

Fatty acids appear in three main types of esters: PLs, TAGs and CEs. These classes are explained concerning their occurrence, synthesis in detail below.

1.1.1 Phospholipids

Every cell in a living system has a barrier that helps keep it protected from the outer environment. This barrier is known as cell membrane or plasma membrane. The membrane forms a border around the cell keeping the cytoplasm, and various organelles inside the cell isolated from the outer environment and allows selected substances such as ions and organic molecules to pass through it making the barrier selectively permeable or semipermeable. The plasma membrane is composed of a lipid bilayer and proteins integrated into the bilayer. The lipid bilayer is important as it acts as a support for different proteins which are involved in important functions viz. signal transduction, DNA replication, protein targeting and cell-cell recognition (Dowhan 1997). Major components of the lipid bilayer are the PL, along with glycolipids and sterols. PLs are amphiphilic molecules that are made of a hydrophilic head part and a hydrophobic tail part (Figure 2).

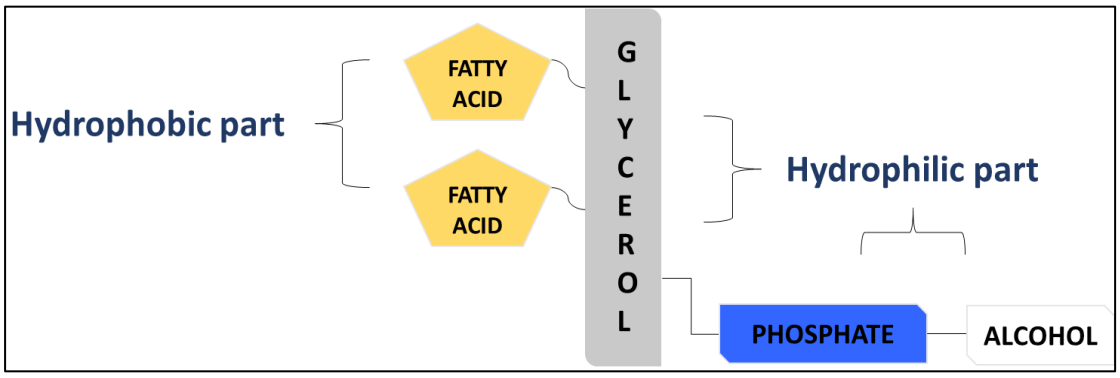


Figure 2: Schematic view of the PL backbone.

PLs, are ubiquitous and are primarily present in the barrier of different cells and organelles in the form of the phospholipid bilayer. Depending on the polar head group attached to the glycerol backbone, there are different types of PL. The major PLs are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and PA (Van Meer, Voelker, and Feigenson 2008). These PLs have acyl chains attached by ester bonds at the *sn-1* and *sn-2* positions of the glycerol backbone. Most eukaryotic membranes

comprise at least/over 50% of PCs. As stated earlier, ER is the leading site for lipid biosynthesis. The PL synthesis takes place in the ER along with conjunction with mitochondria via mitochondria-associated membranes (Van Meer, Voelker, and Feigenson 2008). PA, a minor component of PLs, is the backbone for the synthesis of PL and TAG. Biosynthesis of PA takes place primarily in the ER. The synthesis starts with the addition of acyl-CoA to glycerol-3-phosphate, a rate-limiting step, at *sn*-1 position by glycerol-3-phosphate acyltransferase (GPAT) to produce LPA. The fatty acyl-CoA added at this stage is usually saturated. Further, another acyl-CoA (unsaturated in this case) is added to LPA at *sn*-2 position by acylglycerol-3-acyltransferase (AGPAT) to produce PA.

Following the synthesis of PAs, synthesis of PLs initiates. The biosynthesis of various PLs can take place via two pathways: the Kennedy pathway and cytidine diphosphate diacylglycerol (CDP-DAG) pathway, respectively. The two pathways taking place in mammals are explained in brief below.

- Kennedy pathway: In this pathway, *de novo* synthesis of PE and PC takes place via the CDP-ethanolamine pathway and CDP-choline pathway. It starts with the hydrolysis of the phosphate group from the association of cytosolic phosphatidic acid phosphatase to PA to yield diacylglycerol (DAG) in the ER membrane (Gibellini and Smith 2010). DAG is used in the biosynthesis of both PE and PC subsequently. The CDP-ethanolamine pathway starts with the catalysis of ATP-dependent phosphorylation of ethanolamine by ethanolamine kinase (EK) to produce phosphoethanolamine (Gibellini and Smith 2010). Phosphoethanolamine then reacts with cytidine triphosphate (CTP) to form CDP-ethanolamine via CTP: phosphoethanolamine cytidylyltransferase (ECT) and releases a pyrophosphate (Gibellini and Smith 2010). In the final step, CDP-ethanolamine attaches to the *sn*-3

position of DAG via catalysis of CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase and produces PE. The CDP-choline pathway takes place in a similar manner, with the exception of choline instead of ethanolamine to produce PC (Gibellini and Smith 2010).

- Cytidine-diphosphate diacylglycerol (CDP-DAG) pathway: In this pathway, CDP-DAG synthases catalyse PA to intermediate CDP-DAG, which is used for the synthesis of PI, PS, and cardiolipin. In yeast, PI synthase Pis1 catalyses CDP-DAG to produce PI by replacing cytidine monophosphate (CMP) with an inositol group (Shen and Dowhan 1996; Tamura et al. 2013; Qiang Zhang et al. 2014). In mammals, PS is generated from PC and PE by PS synthases PSS1 and PSS2 which catalyse replacement of choline and ethanolamine with serine (Tomohiro et al. 2009; Vance and Tasseva 2013; Qiang Zhang et al. 2014). Phosphatidylglycerol (PG) is produced by first generating precursor phosphatidylglycerolphosphate (PGP) by PGP synthase, which replaces CMP with glycerol-3-phosphate (Chang et al. 1998; Qiang Zhang et al. 2014). Then, PGP is dephosphorylated by PGP phosphatase PTPMT1 in mammals to produce PG. Cardiolipin is produced via synthesis of PG and CDP-DAG by Cardiolipin synthase CRLS1 (J. Zhang et al. 2011; Lu et al. 2006; Qiang Zhang et al. 2014)

1.1.2 Triacylglycerol

TAGs are esters of glycerol attached to fatty acids where three glycerol hydroxyl groups are esterified. They can be either saturated (solids at room temperature) or unsaturated (liquids – oils at room temperature) fats. Milk has short-chain and medium-chain fatty acids (2-6 carbon and 8-10 carbon) (Beermann et al. 2003), while coconut and palm oils have medium-chain saturated fatty acids. The central

part of animal and plant fatty acids consumed by humans are long-chain fatty acids (16-22 carbon). Adipose tissue is the main site for TAG storage.

- *De novo* synthesis: *De novo* biosynthesis is glycerol-3-phosphate pathway originally shown by biochemist Kennedy where PA again plays an important role (Wendel, Lewin, and Coleman 2010). In this pathway, enzyme acyl-CoA GPAT transfers the acyl group from acyl-CoA to position 1 of glycerol-3-phosphate (now 1-acylglycerol-3-phosphate). While enzyme acyl-CoA 1-acyl glycerol phosphate 2-O-acyltransferase (LPAT) (Coleman 2019) transfers another acyl group from acyl-CoA to position 2 of 1-acylglycerol-3-phosphate by catalysis forming PA. Both these enzymes have two isoforms each in human synthesis pathway. Isoforms of GPAT are present in ER (Poppelreuther et al. 2018) and the outer mitochondrial membrane having both their active sites facing the cytoplasm. Isoforms of LPAT in humans are α and β forms; α form is present in all tissues while the β form is predominant in ER of the cells of heart, liver and pancreas (Coleman 2019). PA is further catalysed by another enzyme called phosphatidate phosphohydrolase to form 1, 2-DAG, which is further catalysed by enzyme DAG transferase to form TAG. DAG transferase enzyme is unique to TAG synthesis, and its activity is high in tissues specific for TAG synthesis.

1.1.3 Cholesterol and Cholesteryl esters

Along with PLs and TAGs, sterols are essential for a eukaryotic system; one function being the formation of the bilayer membrane. Excess of cholesterol can be harmful to the cell. Therefore, excess cholesterol is esterified and stored as CEs in lipid droplets. Cholesterol and CEs are two forms of cholesterol that exist in blood plasma and are present in chylomicrons, Very Low-Density Lipoprotein (VLDL), Intermediate Density Lipoprotein (IDL), LDL, and High-Density

Lipoprotein (HDL) (Gerl et al. 2018). In mammals, synthesis of cholesterol (Figure 3) starts in the cytosol of the cell with the condensation of acetyl-CoA to β-hydroxy- β-methylglutaryl CoA (HMG CoA) by thiolase and then conversion of HMG CoA to mevalonate (6-carbon product) by the ER membrane protein HMG CoA reductase (Yeagle 1985). Mevalonate is then converted to 5-carbon product of isopentenyl pyrophosphate using many steps by cytosolic enzymes (Yeagle 1985). Subsequent reactions give rise to six isopentenyl pyrophosphates. Pyrophosphates condense in the next step to form squalene, a 30-carbon intermediate. Multiple reactions take place using enzymes bound to the ER membrane to convert squalene to cholesterol. Synthesis of cholesterol is tightly regulated in order not to have excess production. If cholesterol is produced in excess, it is esterified using enzyme acyl: cholesterol acyltransferase (ACAT) in ER membrane of intestine and liver (Yeagle 1985). The esterification takes place by esterifying fatty acyl CoA to a hydroxyl group of cholesterol and stored in lipid droplets in the cell. The proportion of sterol to sterol ester differs in different tissues (Yeagle 1985). Cholesterol, which is an important precursor for steroid hormones, is stored in esterified form in the adrenal glands (Yeagle 1985). High amounts of CE found in foam cells are indicators of atherosclerotic disease (Yeagle 1985).

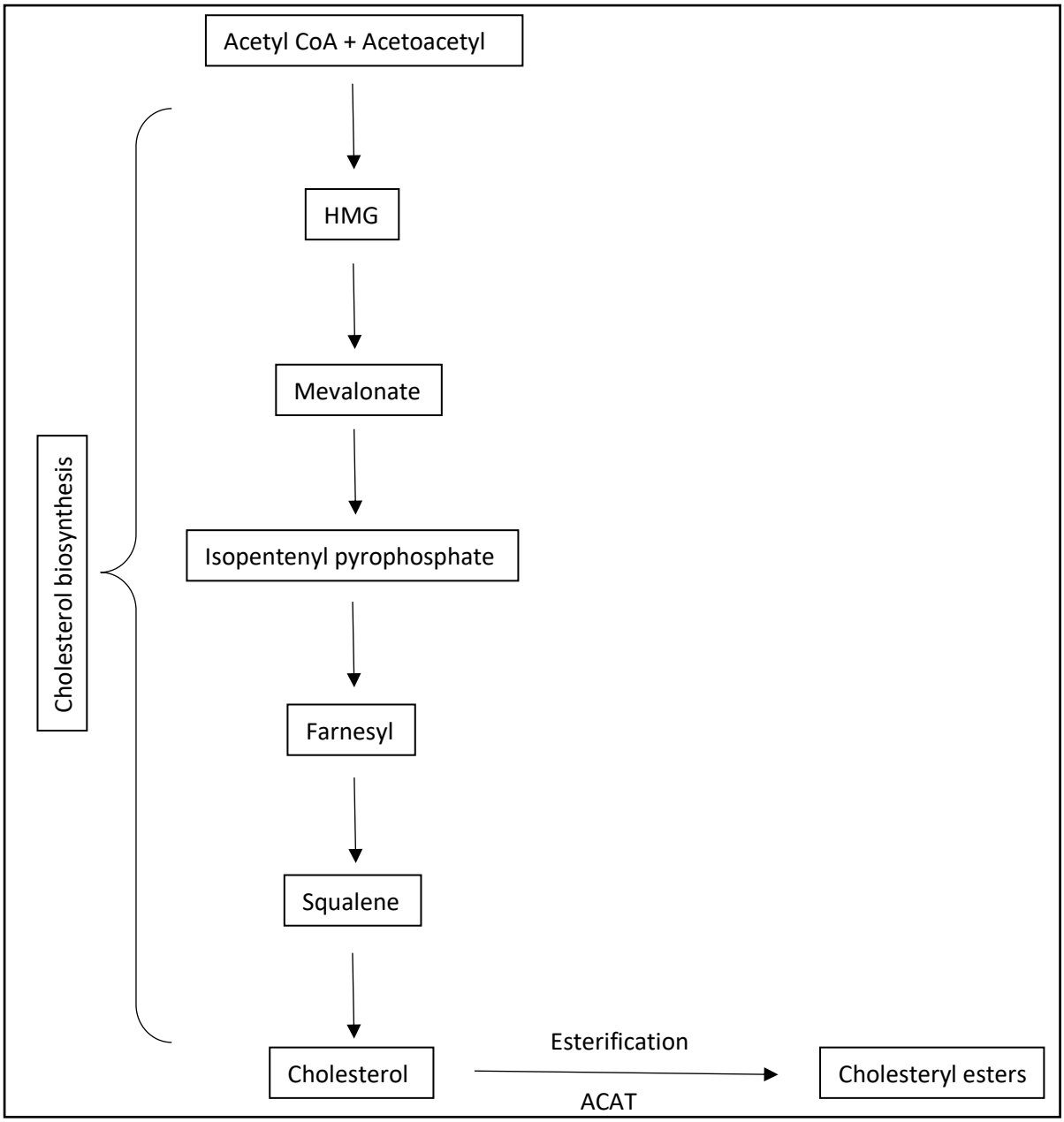


Figure 3: All the steps taking place in the synthesis of cholesterol in cytosol and ER. Excess of cholesterol undergoes esterification using enzyme acyl: cholesterol acyltransferase (ACAT) and is stored in lipid droplets in the cell.

1.2 Lipoproteins and lipid transport

Lipoproteins are biochemical carriers of the insoluble lipids such as cholesterol and TAGs. They are complexes which combine lipids and proteins [referred to as apolipoproteins apo] for the transport of lipids in the body via circulation. The lipids in the blood are mainly fatty acyl esters. Thus, for example, cholesterol, which is insoluble in water, is transported via lipoproteins, which have a hydrophilic surface, in circulation. Lipoproteins consist of two parts; a hydrophobic core made of CEs and TAGs and a hydrophilic surface which consists of free cholesterol, PLs, and specific apolipoproteins. The lipoproteins play an important role in transport and absorption of dietary lipids through the intestine, then from liver to peripheral tissues and vice versa in a mechanism called reverse cholesterol transport. Classification of lipoproteins can be executed based on their lipid composition, size, presence of apos and density. They are of the following types:

1.2.1 Chylomicron and Chylomicron remnants

Chylomicrons originate from the intestine and mainly comprise of a hydrophobic core consisting of TAGs with small amounts of CEs and an outer membrane that has one copy of Apo B-48. Also, the chylomicrons contain small exchangeable apo, such as ApoE and ApoCs. Chylomicrons are secreted from the intestine and transported via the thoracic duct to the bloodstream for the transport of dietary fat to peripheral tissues. This transport is possible because of the exogenous pathway, in which lipoprotein lipase (LpL) enzyme acts on the chylomicrons and catabolizes hydrolysis of TAGs to fatty acids and glycerol. This process gives rise to chylomicron remnants (Pan and Segrest 2016). The size of chylomicrons depends on the amount of fat in the diet (Barter 2014) and is between 75-1200 nm.

After peripheral tissues remove the TAGs, the chylomicrons have CEs with a smaller number of TAGs. These chylomicron remnants then deliver cholesterol to the liver. Their size ranges from 30-80 nm. Chylomicron remnants are atherogenic (Barter 2014).

1.2.2 Very Low-Density Lipoproteins

VLDLs originate from the liver and are responsible for the transport of TAGs from the liver to the peripheral tissues. Like chylomicrons, VLDLs have a hydrophobic core consisting mainly of TAG with small amounts of CEs, an outer membrane and one molecule of Apo B-100 and small exchangeable apo. VLDLs are smaller in size compared to chylomicrons but, like chylomicron remnants, their size (range 30-80 nm) differs according to the amount of TAG present (Barter 2014).

LpL is present at the endothelial surface of capillaries in most of the tissues in the body. The chylomicrons and VLDLs in plasma interact with the LpL, where LpL hydrolyses TAGs and releases free fatty acids which are taken up by various tissues as an energy source. Further, the CE transfer protein transfers CE from HDL to chylomicrons and VLDL (Barter 2014).

1.2.3 Intermediate Density Lipoproteins

After hydrolysis of most of their TAGs, the VLDL remnants have mainly CEs present in their core. They are then called as the IDLs and are in size range of 25-35 nm (Barter 2014). They also have pro-atherogenic properties (Barter 2014).

1.2.4 Low-Density Lipoproteins

LDLs have the size range from 18-25 nm. Like the other lipoproteins, LDLs have a hydrophobic core which mainly consists of CE and small amounts of TAG. A hydrophilic membrane surrounds the core showing the presence of PLs, one Apo B-100 molecule, unesterified cholesterol and small amounts of SMs (Figure 4). As shown in Figure 4 and previously explained by Esterbauer, the surface of LDL particle consists of approximately 700 molecules of PLs, along with one copy of protein Apo B-100 (Esterbauer et al. 1992). The core is composed of around 170 molecules of TAGs, 1600 molecules of CEs, and about one-third of 600 molecules of unesterified cholesterol (Esterbauer et al. 1992; Lund-Katz and Phillips 1984; Hevonoja et al. 2000). Out of the 700 molecules of PLs present in one particle of LDL, 450 molecules are PCs, 185 molecules are SMs, 80 molecules are LPC, around ten molecules are of PEs. In addition, the average particle contains seven molecules of DAGs, and about two molecules of ceramides (Esterbauer et al. 1992; Sommer et al. 1992; Lalanne et al. 1999; Schissel et al. 1996; Amir Ravandi, Arnis Kuksis 1999; Hevonoja et al. 2000). Based on lipid composition present in the core and on the surface of an LDL particle, the particle is in a dynamic state. Therefore, there have been speculations whether the lipids of the core and the surface interact with each other (Ginsburg, Small, and Atkinson 1982; Fenske et al. 1990; Saito et al. 1996; Hevonoja et al. 2000) or are independent (Kroon 1981; Hevonoja et al. 2000). Experiments conducted by Kroon using proton NMR on LDL particles indicate that the core lipids are in a dynamic state, i.e. the composition or structure is not constant, while the surface lipids remain fluid (Kroon 1981; Hevonoja et al. 2000). LpL interacts with chylomicrons and VLDLs by hydrolysing the TAGs via the exogenous pathway (Pan and Segrest 2016). About 20-60% of VLDLs is converted to LDL particles, with the rest being removed from the circulation to the liver (Pan and Segrest 2016). LDL receptors play a big role in the removal of LDL particles from the plasma; therefore, their expression in extrahepatic tissues

and particularly in the liver controls the levels of LDL in circulation. Small dense LDL have high retention time in the circulation. Due to their abundance, the small-sized LDL particles are considered pro-atherogenic compared to large LDL particles as they enter the arterial walls more quickly and are trapped there. This ability leads to them playing an important role in atherosclerosis, and their amount has been shown to be increased in hypertriglyceridemia, type 2 diabetes, and obesity (Barter 2014).

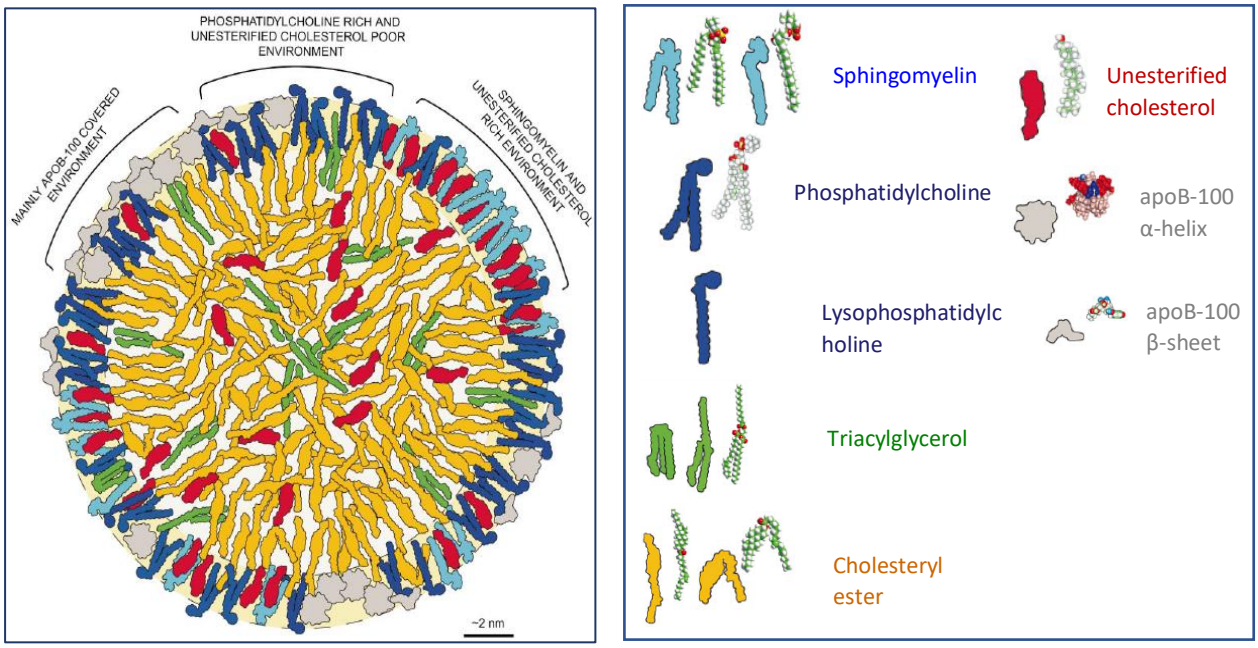


Figure 4: Schematic picture of a cross-section of LDL particle indicating the main lipid types present on the surface and the core of the LDL particle (Hevonoja et al. 2000)

1.2.5 High-Density Lipoproteins

HDLs, like the other lipoproteins, have a hydrophobic core consisting mainly of cholesterol and small amounts of TAGs. The core is surrounded by a hydrophilic layer that is made of unesterified cholesterol, apos, and PLs (Barter 2014). HDLs are the smallest amongst all the lipoproteins present in plasma. Their size ranges from 5-12 nm, and they are also the densest lipoproteins. Apo A-I is the abundant structural protein present on the HDL particle surface. HDL has the possibility of being anti-atherogenic because of its role in reverse cholesterol transport from

peripheral tissues to the liver (Trigatti 2017). As HDL particles are heterogeneous, depending on their size, density, presence of apos, they can be further classified to very high-density HDL, large, medium, and small HDL respectively (Barter 2014).

Apos play an essential role in lipoprotein metabolism. They act as ligands for the binding to lipoprotein receptors, activators, or inhibitors of enzymes during the metabolism of lipoproteins (Wang et al. 2015). Moreover, they form a part of the lipoprotein outer layer and control lipid metabolism. There are different types of apos based on their molecular weight and lipoprotein association (Table 1).

Apolipoproteins	Molecular Weight	Primary Source	Lipoprotein association	Function
Apo A-I	28,000	Liver, Intestine	Chylomicron, HDL	A structural protein of HDL
Apo A-II	17,000	Liver	Chylomicron, HDL	A structural protein of HDL
Apo A-IV	45,000	Intestine	Chylomicron, HDL	Unknown
Apo A-V	39,000	Liver	Chylomicron, VLDL, HDL	Promotes LpL-mediated lipolysis
Apo B-48	241,000	Intestine	Chylomicron	A structural protein of chylomicrons
Apo B-100	512,000	Liver	VLDL, IDL, LDL	A structural protein, a ligand of the LDL receptor
Apo C-I	6,600	Liver	Chylomicron, VLDL, HDL	Activates LCAT
Apo C-II	8,800	Liver	Chylomicron, VLDL, HDL	Co-factor for LpL
Apo E	34,000	Liver	Chylomicron remnants, IDL, HDL	Ligand for LDL receptor
Apo (a)	250,000-800,00	Liver	Lp(a)	Inhibits plasminogen activation

Table 1: Different types of Apolipoproteins. Adapted from the table of Feingold KR, Grunfeld C. Introduction to Lipids and Lipoproteins. [Updated 2018 Feb 2]. In: De Groot LJ, Chrousos G, Dungan K, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-. Available from https://www.ncbi.nlm.nih.gov/books/NBK305896/). LCAT: Lecithin: cholesterol acyltransferase

1.3 Low-Density Lipoprotein Metabolism

The exogenous lipoprotein pathway initiates in the intestine. The pathway leads to the production of chylomicrons and chylomicron remnants (Barter 2014). These lipoproteins help in the organized transfer of various dietary fatty acids for storage and energy in muscle and adipose tissues. Chylomicrons via their hydrolysis and generation of chylomicron remnants allow the transport of dietary lipids into various tissues and the liver. There the lipids will be used for the formation of bile acids, VLDL and further LDL or sent back to the intestine (Abumrad and Davidson 2012; Hussain 2014). The endogenous lipoprotein pathway starts with the formation of VLDL in the liver by the transport of CEs and TAGs to the ER, where they are bound to Apo B-100 (Tiwari and Siddiqi 2012). VLDL synthesis depends on the availability of TAGs. VLDL metabolism takes place in capillaries of peripheral tissues, where TAGs carried in VLDL are hydrolysed by LpL, resulting in the release of fatty acids (Dallinga-Thie et al. 2010). The event gives rise to IDLs which are enriched in CEs and show an affinity for Apo E. Clearance of IDL from circulation takes place via Apo E, which is recognized by LDL receptor-related proteins (LRP) and mediates its uptake (Van De Sluis, Wijers, and Herz 2017; Dallinga-Thie et al. 2010). Only a small amount of IDL is cleared, and in the remaining IDL, the small percentage of TAGs are hydrolysed, an affinity for Apo E changes and formation of LDL takes place (Dallinga-Thie et al. 2010).

In the liver, plasma LDL levels are controlled by the rate of VLDL production and the rate of LDL clearance. Various LDL receptors regulate both processes. The hepatic LDL receptors play an essential role in the regulation of plasma LDL levels. The LDL receptors partially decide the rate of production of LDL from VLDL in a way; for example, a decrease in IDL uptake causes low LDL receptor activity which increases VLDL production and vice versa (Goldstein and Brown

2009). Hepatocyte LDL receptor-mediated endocytosis clears 70% of the circulating LDL (Goldstein and Brown 2009). It undergoes in a manner that when there is an increase in LDL receptors, it leads to a rise in LDL clearance followed by a decrease in circulating LDL levels (Brown, Radhakrishnan, and Goldstein 2018).

LDL receptors have an affinity for Apo B-100 and Apo E and are responsible for the uptake of various lipoproteins along with LDL (Goldstein and Brown 2009). The regulation of LDL receptors depends on the amount of cholesterol present in the cell. The decrease in the levels of cholesterol allows the inactive sterol regulatory element-binding proteins (SREBPs), to convert to active state by transport from the ER to Golgi for the protease cleavage of the SREBP (Goldstein and Brown 2015). SREBPs are transcription factors that are important for the expression of LDL receptors (Goldstein and Brown 2015). Further, the active SREBPs are transported to the nucleus where they allow the transcription of the LDL receptors along with HMG-CoA reductase, a rate-limiting enzyme participating in the cholesterol synthesis. Conversely, high levels of cholesterol in the cell keeps the SREBPs in the inactive state in the ER, which does not allow the transcription of LDL receptors, elevating the levels of LDL particles in circulation (Goldstein and Brown 2009). PCSK9, a ubiquitously expressed protein, is also involved in controlling the amounts of hepatic LDL receptors (Horton, Cohen, and Hobbs 2007). When this protein binds to the LDL receptor, it causes its degradation of the receptor in the lysosomes and prevents the recycling of the receptor to the plasma membrane for the uptake of LDL particles (Horton, Cohen, and Hobbs 2007). Blockage of this protein by a mutation in the functional domain of the protein upregulates the amount of cell surface LDL receptors and lowers the of LDL particle concentration in plasma (Horton, Cohen, and Hobbs 2007).

1.4 Cardiovascular diseases

CVDs are a group of diseases that are related to heart and blood vessels. Each disease type has a different mechanism of action. This group consists of diseases such as cardiomyopathy, congenital heart disease, hypertensive heart disease, atherosclerosis, aortic aneurysms (Nascimento et al. 2018). CVDs are the number one cause of mortality worldwide in men and women, accounting for about 80% of the deaths in middle and low-income countries (Phillips and Guazzi 2015; Nascimento et al. 2018). Various risk factors that play a key role in the onset of CVD include alcohol, unhealthy diet, obesity, high fasting plasma glucose, high cholesterol, diabetes, impaired kidney functions and high blood pressure (Phillips and Guazzi 2015). Maintaining proper lifestyle and avoiding different risk factors responsible for the CVD, can help improve the health or prevent the diseases.

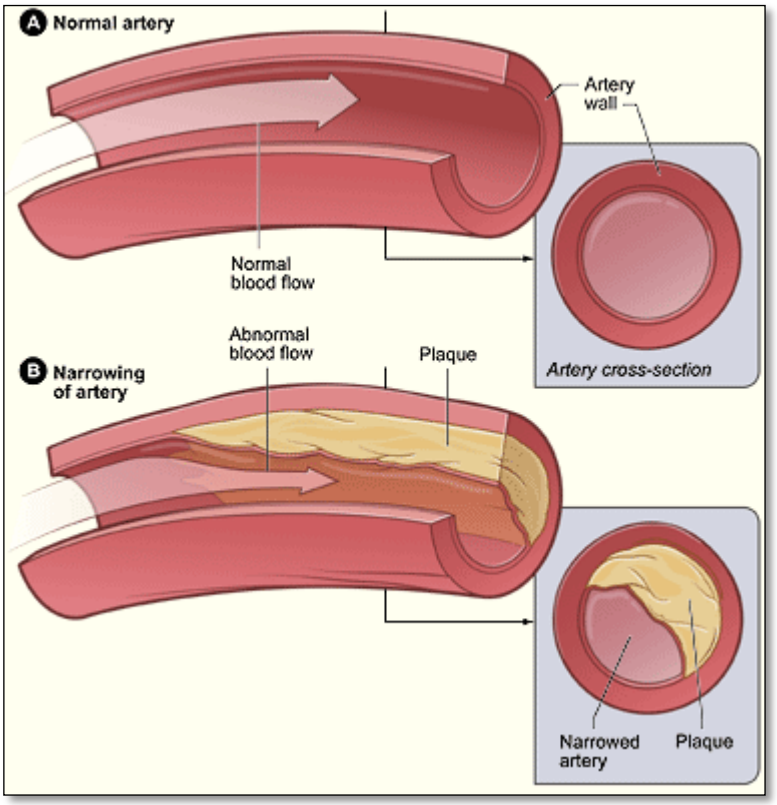


Figure 5: A) Shows the inside of a healthy artery where blood flows without any hindrance, B) shows deposition of cholesterol and other lipids slowly narrowing the arteries and diminishing the blood flow—adapted from www.nhlbi.nih.gov/health-topics/atherosclerosis.

1.4.1 Atherosclerosis

Atherosclerosis is a type of chronic CVD that is characterised by the deposition of cholesterol in large and medium-sized arteries (Figure 5). The build-up of atherosclerotic plaques leads to the blockage of arteries, which increases the risk of stroke and heart attack (Herrington et al. 2016). Depending on the arteries affected by the plaque formations, atherosclerosis-related diseases such as coronary heart disease (arteries supplying oxygen-rich blood to the hear are narrowed due to build-up of plaques), carotid artery disease (plaques forming in the carotid arteries on both sides of the neck), peripheral artery disease (plaques formation in arteries carrying blood to arms and legs) and chronic kidney disease (plaque formation in the renal arteries) has the possibility to occur (www.nhlbi.nih.gov/health-topics/atherosclerosis).

An arterial wall is made up of three different layers, intima, media, and adventitia. The innermost layer, intima, is separated from circulation by a layer of endothelial cells, the middle layer, media, consists largely of smooth muscle cells and the outer layer, adventitia, of fibroblastic cells. The inner endothelial cell layer forms as a barrier between the blood and the arterial wall. Lipoproteins are normally fluxed in and out of the arterial wall. While crossing through the endothelial barrier, some of these ApoB-lipoproteins are retained in the arterial intima and accumulation of these particles is initiated along with their exposure to local enzymes which modify them (Tamminen et al. 1999; Carew 1989; Borén and Williams 2016). Inflammation and accumulation of these modified lipoproteins in the form of lipid droplets or vesicles in the intima mark the onset of atherosclerosis (Tîrziu et al. 1995; Hevonoja et al. 2000). This accumulation triggers the immune cells to be migrated in the intima, therefore, encountering the accumulated LDL particles and transforming into foam cells. The process of recruitment of more immune cells is started via the secretion of cytokines by the

transformed foam cells, which starts the process of plaque formations (Simmons et al. 2016). The formation of plaques is, therefore, the next step in the progression of atherosclerosis (Simmons et al. 2016).

1.4.2 Inflammation in atherosclerosis

In a healthy blood circulation, the arterial endothelium, which is in contact with the blood, does not allow the adhesion of leukocytes (Libby 2002). As mentioned above, the accumulation of lipids in the intima induces endothelial activation, and the process is initiated by recruitment of leukocytes to the intima via the endothelial-leukocyte adhesion molecules (Poole and Florey 1958; Libby 2002). One type of endothelial-leukocyte adhesion molecule that has been studied is the vascular cell adhesion molecule-1 (VCAM-1) which recruits leukocytes such as monocytes and T-lymphocytes (Figure 6) (Libby 2002). The VCAM-1 molecules are expressed in the parts of endothelium that are prone to lesion formation, and their initiation depends on inflammation inflicted by modified lipoproteins accumulated in the intima layer (Cybulsky and Gimbrone 1991; Libby 2002). Modification in lipoproteins (for example by oxidation) allows activation of the VCAM-1 gene mediated by nuclear factor-kB, and interleukin (IL)-1β. This process initiates the expression of VCAM-1 in the endothelium lesion-prone areas of the arteries (Collins and Cybulsky 2001; Libby 2002).

On the other hand, endothelial regions prone to less atherosclerosis possess shear-stress response elements. They are superoxide dismutase and nitric oxide synthase. They inhibit the VCAM-1 gene expression and hence inhibit leukocyte infiltration (Topper and Gimbrone 1999; De Caterina et al. 1995; Libby 2002). After the attachment of leukocytes to the endothelial cells, the process of diapedesis causes entry of leukocytes in the intima (Libby 2002). The leukocytes

are recruited in the intima of the arteries by various chemoattractant cytokines. One example of the cytokine is monocyte chemoattractant protein-1 (MCP-1) (Libby 2002). Research conducted in multiple laboratories shows less accumulation of the monocytes and lipids in MCP-1- and LDL receptor gene-deficient mice (Gu et al. 1998; Landin Boring, Jennifa Gosling and Charo 1998; Libby 2002). Following the entry of the monocytes in the intima, the monocytes undergo morphological changes to form macrophages. The macrophages express scavenger receptors such as CD36 and scavenger receptor A (SRA) that take up the retained and accumulated modified lipoproteins (such as LDL, VLDL) to give rise to foam cells – a key feature of arterial lesions of atherosclerosis (Libby 2002). Foam cells give rise to the formation of fatty streak in the affected arteries, which can later evolve in a complex lesion. Rupture of an atherosclerotic plaque then leads to clinical symptoms later in life (Libby 2002). The fatty streak advances to atheromas and complicated lesions via multiplication of smooth muscle cells, which get accumulated in plaques making the lesions bulkier. Formation of lesions may lead to the narrowing of the arteries causing the plaques to disrupt physically, which will activate thrombosis (Davies 1996; Libby 2002). Formation of new blood vessels from the pre-existing vasculature in angiogenesis and atheromas can develop in these new blood vessels as microvascular channels (Libby 2002). Physical disruption of plaques occurs in three different ways; superficial erosion of endothelial cells, disruption of microvessels formed in atherosclerotic plaques and a fracture in the fibrous cap of the plaques, which the most common and studied way (Virmani et al. 2002; De Boer et al. 1999; Libby 2002).

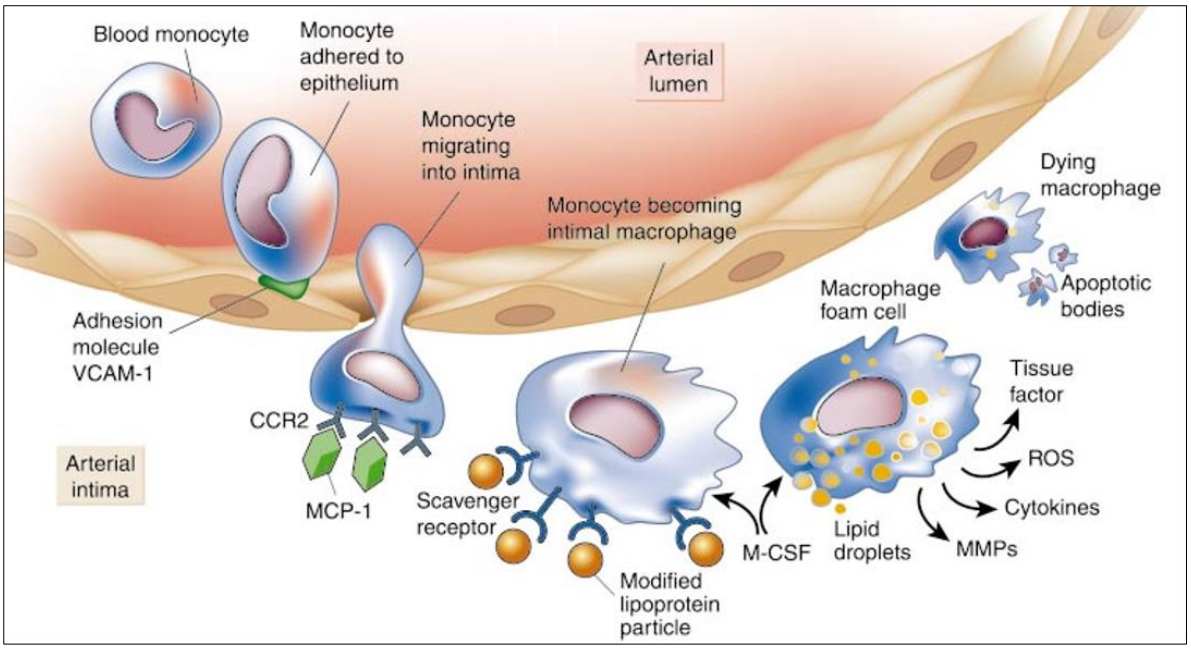


Figure 6: Schematic figure of recruitment of monocytes to the arterial intima to form atherosclerotic plaques and cause inflammation. Figure adapted from(Libby 2002).

1.5 Effect of diet

A balanced diet is an essential aspect for a healthy lifestyle and the proper functioning of our body. But what defines a balanced diet? What are the elements to be considered for a balanced diet? There have been a lot of diet-based studies carried out throughout the world to understand which factors are important for the appropriate supply of nutrients for a healthy individual. Previous research shows that diets which are rich in PUFAs help reduce blood LDL levels with an increase in HDL levels and reach a balanced fat consumption without any harmful effects (Feldman 2002; Egert and Stehle 2011; Njike et al. 2015).

One of the most studied diet types is the Mediterranean diet. The Mediterranean diet primarily consists of different fruits, vegetables, usage of olive oil, dry fruits (nuts), fish and limited consumption of alcohol (Widmer et al. 2015b). A randomized controlled trial study performed using the Mediterranean diet showed

patients consuming the diet had a lower number of monocytes and positive regulation of genes involved in LDL-oxidation in cardiovascular diseases (Trial et al. 2006; Salas-Salvadó et al. 2011; Widmer et al. 2015a). Fruits and vegetables are natural sources of various nutrients. Any type of diet suggested by nutritionists includes a certain amount of fruit and vegetable intake. Studies show that the consumption of fruits and vegetables decreases the risk of CVD because of the low intake of calories and also the presence of micronutrients (Widmer et al. 2015b). Another cross-sectional study shows that consumption of fruits and vegetables is associated with lower body mass index (Lin and Morrison 2002; Widmer et al. 2015a). A large meta-analysis study of over 200,000 patients showed 17% reduction of CVD in patients that were instructed to ingest three to four servings of fruits and vegetables every day during the study period, which was rechecked after two years of time period (Dauchet, Amouyel, and Dallongeville 2009; Widmer et al. 2015a). The American Health Association (AHA) and European Society of Cardiology (ESC) suggest that consumption of fats through the use of vegetable oils instead of saturated and trans fats as oil may have antioxidant properties, free radical scavengers and enzyme modulators (Lichtenstein et al. 2006; Graham et al. 2007; Fuhrman and Aviram 2001; Widmer et al. 2015a). In a crossover diet study, Marin and her group showed that diet rich in olive oil helps to improve endothelial function and reduce inflammation along with improving the endothelial progenitor cell numbers significant for arterial endothelial repair (Marin et al. 2011; Widmer et al. 2015a). In the study conducted by van Horn, the data suggest that the intake of whole-grain foods is associated with decreased CVD morbidity and mortality by lowering the plasma lipid levels (Van Horn 1997; Widmer et al. 2015a). The AHA guidelines recommend dietary fibre intake of 25-30 g per day (Van Horn 1997; Widmer et al. 2015a). A study conducted by Schwab and colleagues in 2018 showed that *Camelina sativa* oil, a plant-based source improves the serum

lipid profile in subjects with impaired fasting glucose metabolism (U. S. Schwab et al. 2017).

Diet studies have previously shown that walnuts are a rich source of ALA and have a high content of PUFAs, which have cardiovascular benefits (Feldman 2002; Damasceno et al. 2011; Njike et al. 2015). A review article by Feldman suggests that eating 2-3 walnuts per day reduces total cholesterol and LDL cholesterol levels (Feldman 2002; Njike et al. 2015).

Fish and fish oil supplements are a rich source of long-chained n-3 PUFAs and are consumed regularly in many countries. AHA guidelines recommend consumption of fish twice per week as it protects from dysrhythmia and CVD (Graham et al. 2007; Widmer et al. 2015a). Fish-based diet studies show that consumption of fish has beneficial effects on CVD and that it is associated with improved lipid profiles (Balk et al. 2006; Widmer et al. 2015a). Studies show that n-3 EPA and DHA found in fish and fish oil supplements have anti-inflammatory effects and that they associate with reduced risk of recurrent coronary artery diseases (Bouwens et al. 2009; Kris-Etherton, Harris, and Appel 2002; Swanson, Block, and Mousa 2012). DHA plays a vital role in the development of the foetal brain and eyesight (Birch and O'Connor 2001; Lyberg, Adlercreutz, and Adlercreutz 2005). A large cohort study conducted in Japan comparing participants that have impaired glucose metabolism with normoglycemic participants showed that supplementing EPA to the patients having impaired glucose metabolism elicited lower coronary artery events compared to patients with impaired glucose metabolism not given EPA (Oikawa et al. 2009; Swanson, Block, and Mousa 2012). Consumption of fish is relatively high in the Japanese population, and so providing EPA treatment showed more cardiovascular benefits in the study participants (Oikawa et al. 2009; Swanson, Block, and Mousa 2012).

2 Aim

This study was part of a randomized controlled trial conducted with 41 human volunteers for 12 weeks. The study used samples from two fish diet groups (fatty fish = 20 and lean fish = 21). The aim of this study was to analyse changes caused to human LDL particles from consumption of these two types of fish diet. Blood plasma samples were collected before and after consumption of the fish diets. These samples were **(1)** analysed to study if consumption of fish diet causes any changes to the surface of the lipidome of LDL particle. The samples were also **(2)** analysed to study changes caused to the composition of the core of the LDL particles.

3 Materials and Method

A total number of 41 volunteers with impaired fasting glucose, having body mass index of 25-36 kg/m^2, and belonging to the age group of 43-72 years, had participated in a dietary study where they consumed experimental diets for 12-weeks. This study was a part of a randomized controlled trial (U. S. Schwab et al. 2017). The participants were randomly divided into two groups, where they were provided either with fatty fish diet (4 fish meals/week) or lean fish diet (4 fish meals/week). The volunteers were recruited in Kuopio by advertising about the trial in newspapers and by contacting volunteers who had previously participated in dietary experiments. Primary criteria for the participants were fasting plasma glucose concentration of 5.6-6.9 mmol/l, ≤11.0 mmol/l value of 2-h glucose concentration in the oral glucose tolerance test (OGTT), fasting serum total cholesterol concentration to be ≤7.0 mmol/l, along with LDL cholesterol and total TAG to be ≤5.0 mmol/l and ≤4.0 mmol/l respectively (U. S. Schwab et al. 2017). Blood plasma samples of 41 individuals were available for the isolation of LDL lipoproteins. LDL was isolated from samples that were taken before and after the consumption of the fish diets. Extraction of the LDL lipids was carried out using

the Folch method (Folch, Lees, and Sloane Stanley 1987), and the lipid species profiles were recorded by a triple quadrupole MS using tandem mass spectrometry (MS/MS) scans. The following flowchart (Figure 7) gives a brief overview of the experimental setup.

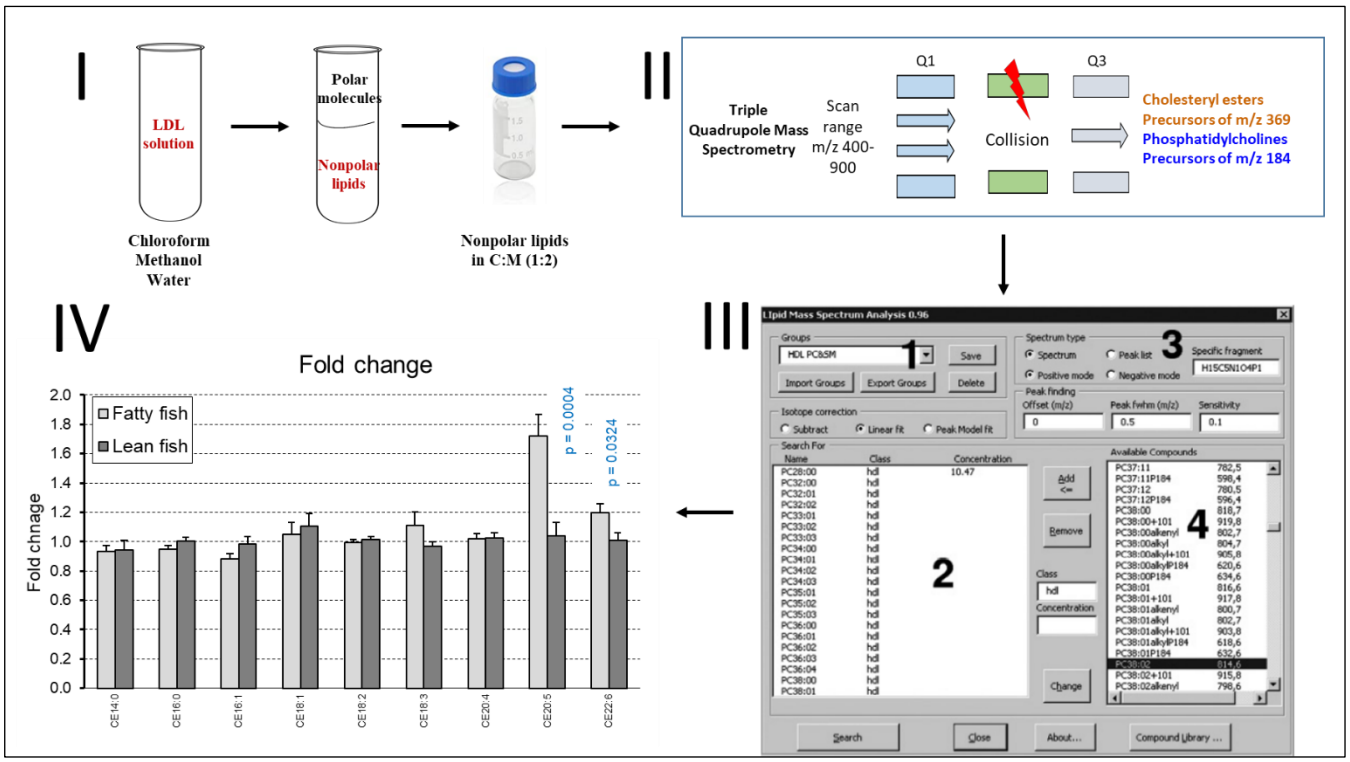


Figure 7**:** Flow chart of the lipid analysis: I) Folch extraction of lipids, II) Mass spectrometric detection by employing lipid class-specific scans (e.g., CE [*m/z* 369] and PC [*m/z* 184]), III) LIMSA analysis of the spectral data of phosphatidylcholines, IV: Bar graph profiles treated with statistics.

3.1 Extraction of Lipids

The Folch method is a traditional and straightforward extraction method of choice which uses chloroform and methanol for isolation and purification of dissolved lipids from a sample (Folch, Lees, and Sloane Stanley 1987). It was used for the first time by Jordi Folch in 1957 for isolation of brain lipids from animal tissues. In the current experiment, the accurate volumes of the blood plasma LDL were recorded to be able to standardize the concentrations of the final extracts, which were later infused into a mass spectrometer. In the Folch extraction, the starting solution requires 450 µl of the aqueous solution. For example, if 190 µl of LDL

solution was taken into a Kimax tube, then 260 µl of millipore-quality water was added. Then 3 ml of methanol (M) was added, and the solution is vortexed. Next 6 ml of chloroform (C) was added to the same solution, and the solution was vortexed again and kept in the dark for 30 minutes. After this incubation, the solution was centrifuged for 10 minutes at 3000 rpm, and the clear supernatant was collected into another tube. This step was performed for the complete removal of protein mass from the solution. Then 1.8 ml of Millipore water was added, and the solution was vortex mixed and centrifuged for 10 minutes at 3000 rpm. Addition of water to the C: M containing supernatants water created a two-phase system. The upper phase contained polar molecules and the lower phase the nonpolar lipids. The lower phase was transferred into another Kimax tube. 4.5 mL of theoretical lower phase (chloroform/methanol/water, *i.e.* C/M/W, 86/14/1 by vol) was added to the remaining upper phase from the original solution, and that solution was vortex mixed. That solution was centrifuged for the second time for 10 minutes at 3000 rpm, and its lower phase was transferred to the previous lipid extract. The upper phase was discarded. The lower phase was evaporated into dryness using nitrogen gas. As soon as the solution was evaporated to near dryness by nitrogen flow, 2 ml of C: M 1:2 solution was immediately added. Finally, the solution was divided into two silanized vials containing 1 ml in each vial and kept in -80 degree Celsius.

3.2 Determination of lipid profile

Determination of the lipid species composition in the sample extracts was performed by using a triple quadrupole MS (Agilent 6490 Triple Quad LC/MS with iFunnel technology, Agilent Technologies Inc.) (Figure 8). MS is used for analysing molecular based on their mass to charge (*m/z*) ratio. In MS, the ions are scanned based on their *m/z* ratio, and the intensity of each *m/*z ratio value is determined with the amount of ions reaching the detector.

For the mass spectrometry of lipids, each stored sample was brought into room temperature, and a mixture with standardized liquid concentration was used to be infused into the MS equipment. The total volume of the solution to be infused in the MS equipment was 300 µl. The aliquot of the lipid extract of a sample that was included depended on the original volume of lipoprotein solution available for the lipid extraction (the more LDL added in extraction, the less extract was required for the infused solution). If the original volume of the LDL sample taken were 190 µl, then 38 µl of its lipid extract would be used, and the remaining would be C: M (1:2). Since the original volumes used for many of the LDL samples were less than 190 µl, the formula: V lipid extract = (190 µl/V original LDL) x 38 µl was used to calculate the accurate amount of lipid extract to be used for the infusion solution. After the mixing of sample extract and C: M, 4 µl of SPLASH LipidoMIX^{TM} Internal Standard – [Avanti Polar Lipids, Inc., Alabaster, AL, USA, the components utilized for lipidome analyses were PC15:0-18:1(d7), PE15:0-18:1(d7), LPC18:1(d7), CE18:1(d7), TAG15:0-18:1(d7)-15:0 and SM18:1-18:1(d9)], 4 µl of Ceramide/Sphingoid Internal Standard Mixture I and 20 µl of ammonium solution were added just before infusion. In the experiment, the samples were infused by a syringe pump into the MS equipment and passed through an ion source, where ionisation of the sample took place (Figure 9-1). The next step was passing the ions through a mass analyser, where the ions were scanned with MS+ scan or with specific scan modes for each lipid class. In precursor ion scans, all of the ions (also called precursor ions) of the sample were scanned, fragmented and then the fragmented ions (also called product ions) were selected based on lipid class-specific scans and sent to the detector for detection [Figure 8-(2-5)]. Precursor ion scans of *m/z* 184 for PC, SM, LPC, and *m/z* 369 for CE were used (Brügger et al. 1997; Duffin et al. 2000). TAGs were analysed from the MS+ scan as ammonium adducts (Duffin, Henion, and Shieh 1991). The scan range was arranged to *m/z* 400-900 (Padro et al. 2015). Further, the mass

spectra of the samples were collected and analysed with statistical and bioinformatics tools.

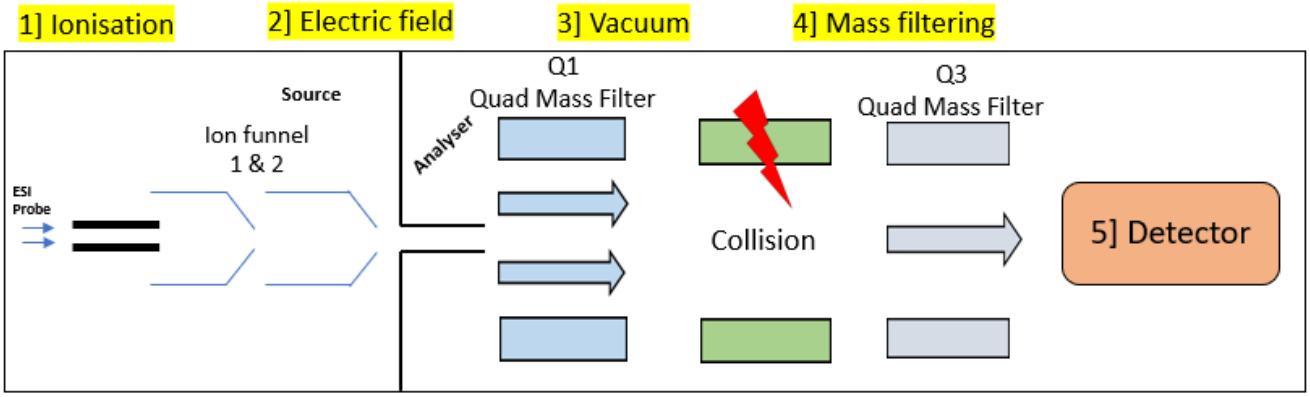


Figure 8: General schematic of triple quadrupole ESI-MS (Electrospray Ionisation Mass spectrometer).

3.3 Data analysis and statistics

Following the MS recording of the lipid profiles, the data were processed by LIMSA software (Haimi et al. 2006), which was used to identify the lipid species and calculate their concentrations based on the internal standards. The LIMSA software was running as an "add-in" in Microsoft Excel version 2003. In the software, there are options where the user can customize the parameters of peak detection depending on the spectral characteristics of the samples. Followed by setting these parameters, relevant lipids species, potentially available in the samples, were selected from the programme lipid library, and the internal standards were defined with their known concentrations. If a lipid species is missing in the database of LIMSA, there is the option to add those species manually through the "Compound Library" interface by telling the structure atom by atom. For the analysis of the data, each MS or MS/MS scan was processed separately and saved as an Excel file. Finally, every Excel file contained only certain lipid classes, which allowed to focus on and resolve the species profiles

each lipid class at a time. Changes within the group were analysed using paired Student´s t-test, and $p<0.05$ was regarded as statistically significant. Further fold change was also calculated by dividing the final relative concentration of each sample lipid (week 12) with the relative concentration of each sample lipid at baseline (week 0).

4 Results

Blood plasma-derived LDL particles from 41 individual volunteers were studied for lipid composition. Total of 20 individuals belonged to the fatty fish group and 21 to the lean fish group, and they all were sampled for blood before and after having the experimental diets, four fish meals/per week, for 12 weeks. At first, lipid class profiles were studied and then the lipid species profiles in each lipid class were examined. When reporting the possible changes in these lipid class and lipid species profiles, the surface membrane lipids were addressed first and then the lipids in the core of the LDL particles.

The LDL particles were characterized by high CE contents (group averages ranging between 76 and 79 mol% per total lipid), and the other major neutral lipid class was the TAGs (group averages 5 – 6 mol%) (Fig. 9). The main PL class was PC (11 – 13 mol% of total lipids), and the SM (4 – 5 mol%) and LPC (1 mol%) were the other detected classes. The lipid class composition of the LDL particles did not change due to the fatty fish or the lean fish diet (Fig. 9A, B). In addition, the fold change values for the major lipid classes, LPC, PC, SM, TAG and CE remained similar in the fatty and lean fish groups (Fig. 9C). The most evident change, although not statistically significant ($p = 0.0512$), was found for the TAG in the fatty fish group (Fig. 9A). The fatty fish diet decreased the proportion of TAG from 6.1mol%, detected before the feeding trial, to 4.9 mol%, after the trial.

When only the surface PLs of the LDL particles were addressed, the primary lipid class was PC (group averages ranging between 69 and 70 mol% per total PL), and the remaining marked PLs classes were SM (24 – 25 mol%) and LPC (6 – 7 mol%) (Fig. 10). The PL class composition of the LDL particles did not change due to the fatty fish or the lean fish diet (Fig. 10A, B). Besides, the fold change values for the PL classes, PC, SM and LPC remained the same in the fatty and lean fish groups (Fig. 10C).

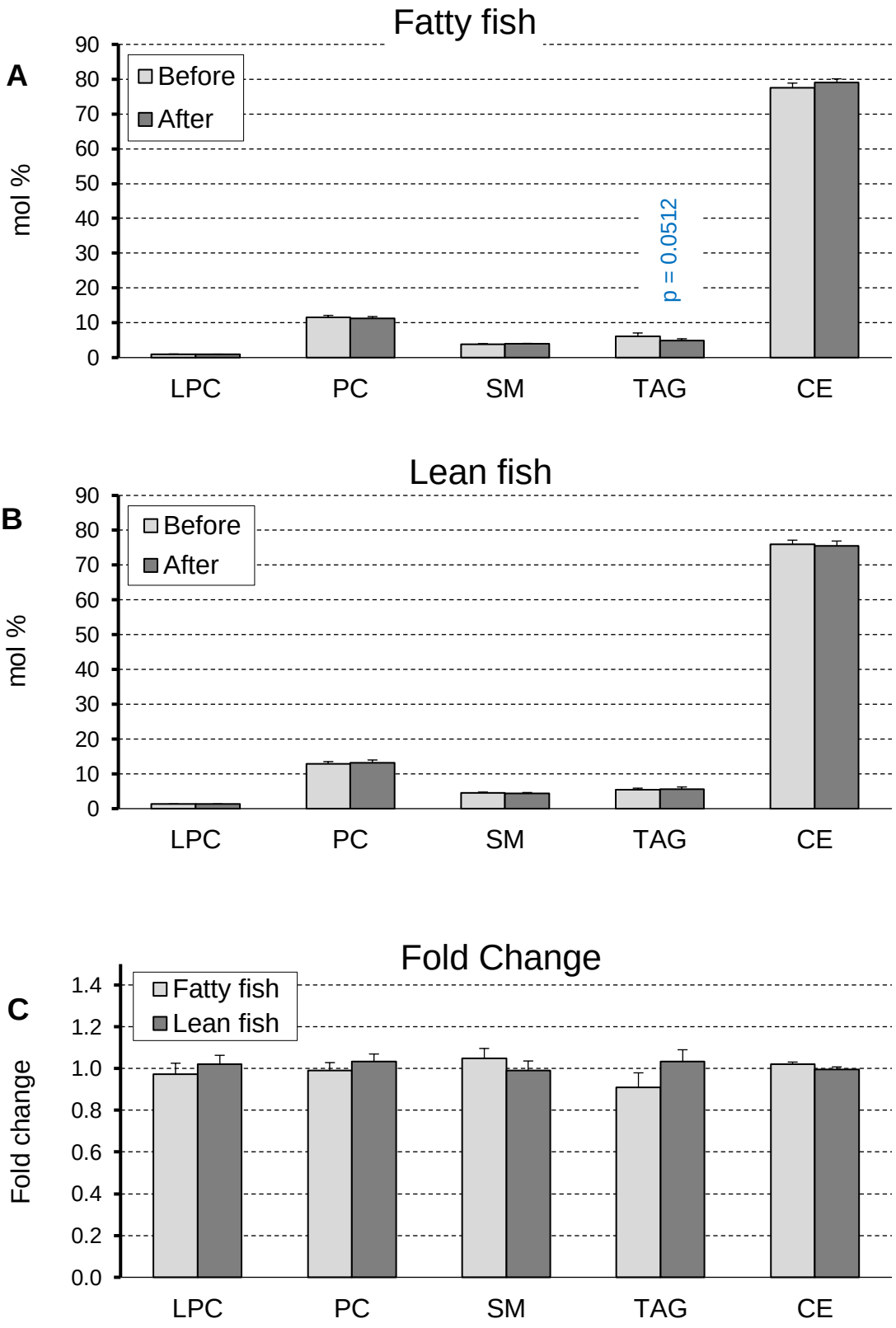


Figure 9. Lipid class profile in fatty fish (A) and lean fish (B) groups before and after the experimental diet and (C) the fold change (mol% after 12 weeks/mol % at day 0) in both dietary groups. Statistical significance (p-value in blue) refers to Student´s paired t-test.

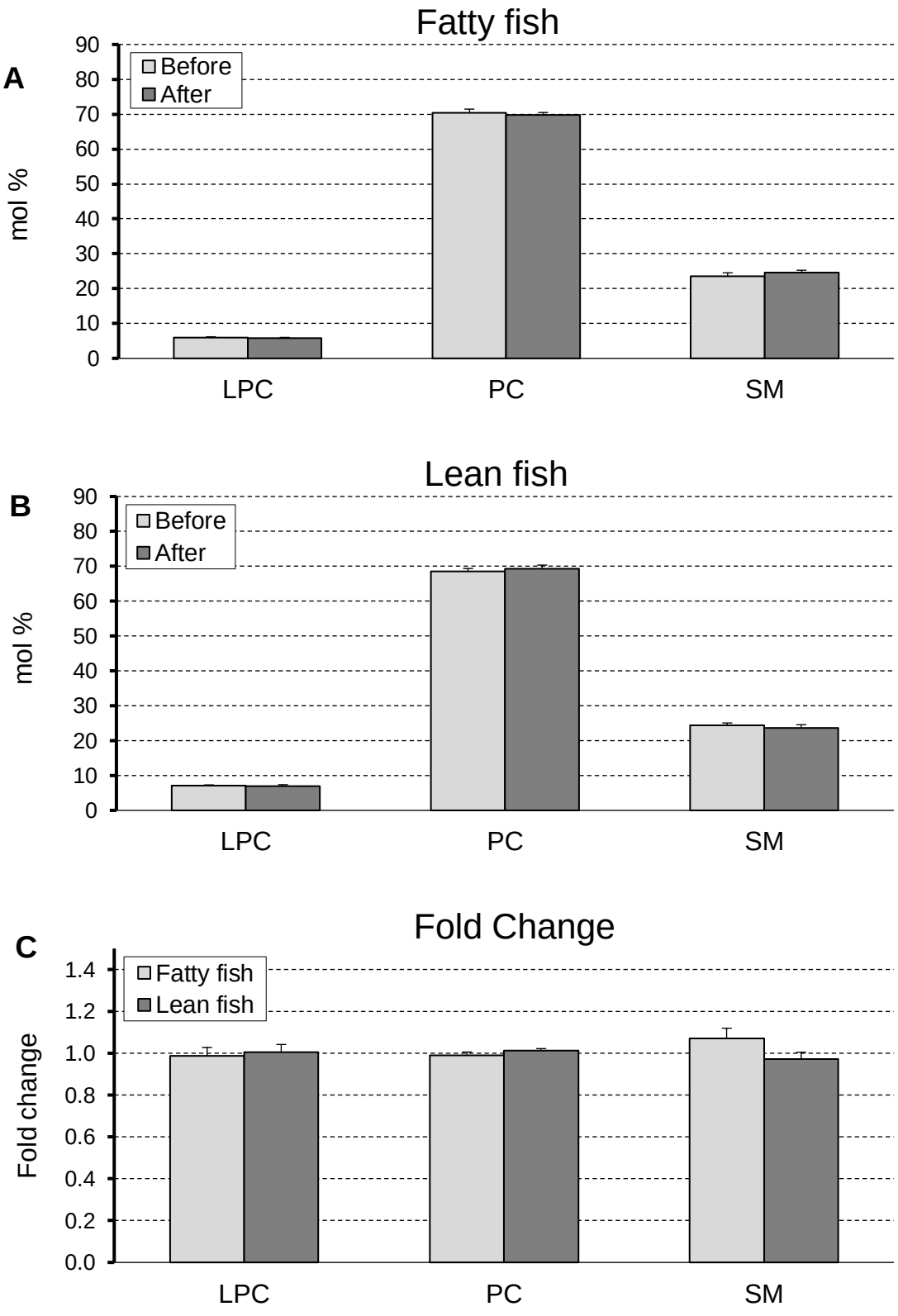


Figure 10. Phospholipid class profile (LPC, PC, and SM) of LDL particle surface in fatty fish (A) and lean fish (B) groups before and after the experimental diet and (C) the fold change (mol% after 12 weeks/mol % at day 0) in both dietary groups. No statistical significances (Student´s paired t-test).

When the PCs of the LDL particles were addressed, the major lipid species observed in both the fatty fish and lean fish groups were PC 34:2 (averages between 27 and 28 mol% per total PC), PC 36:2 (averages between 14 and 15 mol% per total PC), PC 34:1 (averages ranging between 13 and 15 mol% per total PC), PC 36:4 (averages between 8 and 8.8 mol% per total PC) PC 36:3 (averages between 6 and 7 mol% per total PC), and PC 38:6 (averages between 5 and 6 mol% per total PC) (Fig. 11). In addition to the conventional acyl chains bonded by ester bonds, we detected a unique class of PLs called ether lipids which have alkyl chains attached at the *sn-1* position of the glycerol backbone by an ether bond (marked as PC 38:6e, for example). The PC species composition of the LDL particles showed significant changes due to the fatty fish and the lean fish diet (Fig. 11A, B). In Fig. 11A, significant increase in PC species of PC 36:5 ($p = 0.0196$), PC 38:6 ($p = 0.0105$), PC 38:6e ($p = 0.0082$), and PC 40:6 ($p = 0.0062$) were observed. Along with the increase in the above-mentioned PC species, a significant decrease was also found in PC 36:3 ($p = 0.0340$). The significant changes found in lean fish group were decreased in the mol% of species of PC 32:1 ($p = 0.0377$), and PC 40:5 ($p = 0.0386$). Also, the fold change values for the PC showed changes in the fatty and lean fish groups as well (Fig. 11C). Statistical significances were found with increase in species PC 32:0e ($p = 0.0330$), PC 38:6 ($p = 0.0041$), PC 40:6 ($p = 0.0032$), and PC 40:6e ($p = 0.0098$). Clear changes, although not statistically significant were found in two species; PC 36:5 ($p = 0.0519$) and PC 38:6e ($p = 0.0552$) in the fold change values.

Another surface lipid class of the LDL particles addressed was the SM. Major lipid species found in both the fatty fish and lean fish groups were SM 16:0, SM 18:0, SM 24:1, and SM 24:2 (Fig. 12A, B). These lipid species comprised more than 5 mol% of total SM. The SM lipid species composition of the LDL particles did not change due to the fatty fish or the lean fish diet (Fig. 12A, B). Similarly,

the fold change values for the SM lipid species remained the same in the fatty and lean fish groups with minor changes (Fig. 12C).

The major lipid species found in LPC class in both fatty fish and lean fish groups were LPC 16:0, LPC 18:0, LPC 18:1, and LPC 18:2 (Fig. 13A, B). Their proportions remained similar in both groups, but significant changes found were in minor species from the fatty fish group such as LPC 20:5 ($p = 0.0295$), and LPC 22:6 ($p = 0.0387$). The LPC lipid species composition of the LDL particles did not change due to the lean fish diet (Fig. 13B). However, a statistically significant change was found in LPC 22:6 ($p = 0.0308$) (Fig. 13C) in the fold change values.

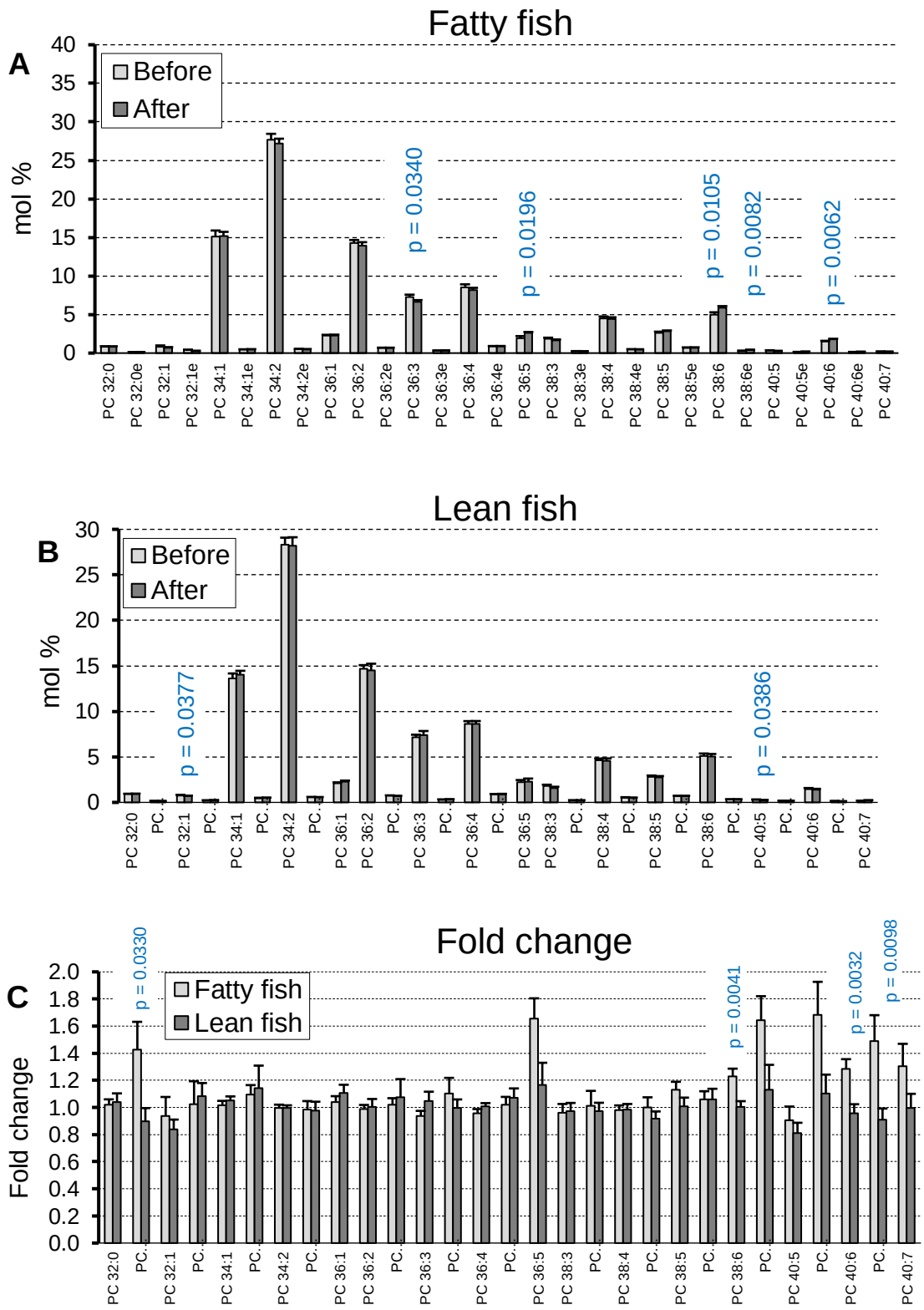


Figure 11: PC (PC = diacyl species, e = species with one acyl and one alkyl chain) species lipid profile in fatty fish (A), lean fish (B) groups before and after the experimental diet and (C) the fold change (mol% after 12 weeks/mol % at day 0) comparison of the two dietary groups. The acyl chain is abbreviated [chain length]: [number of double bonds]. Statistical significance (p-value in blue) refers to Student´s paired t-test.

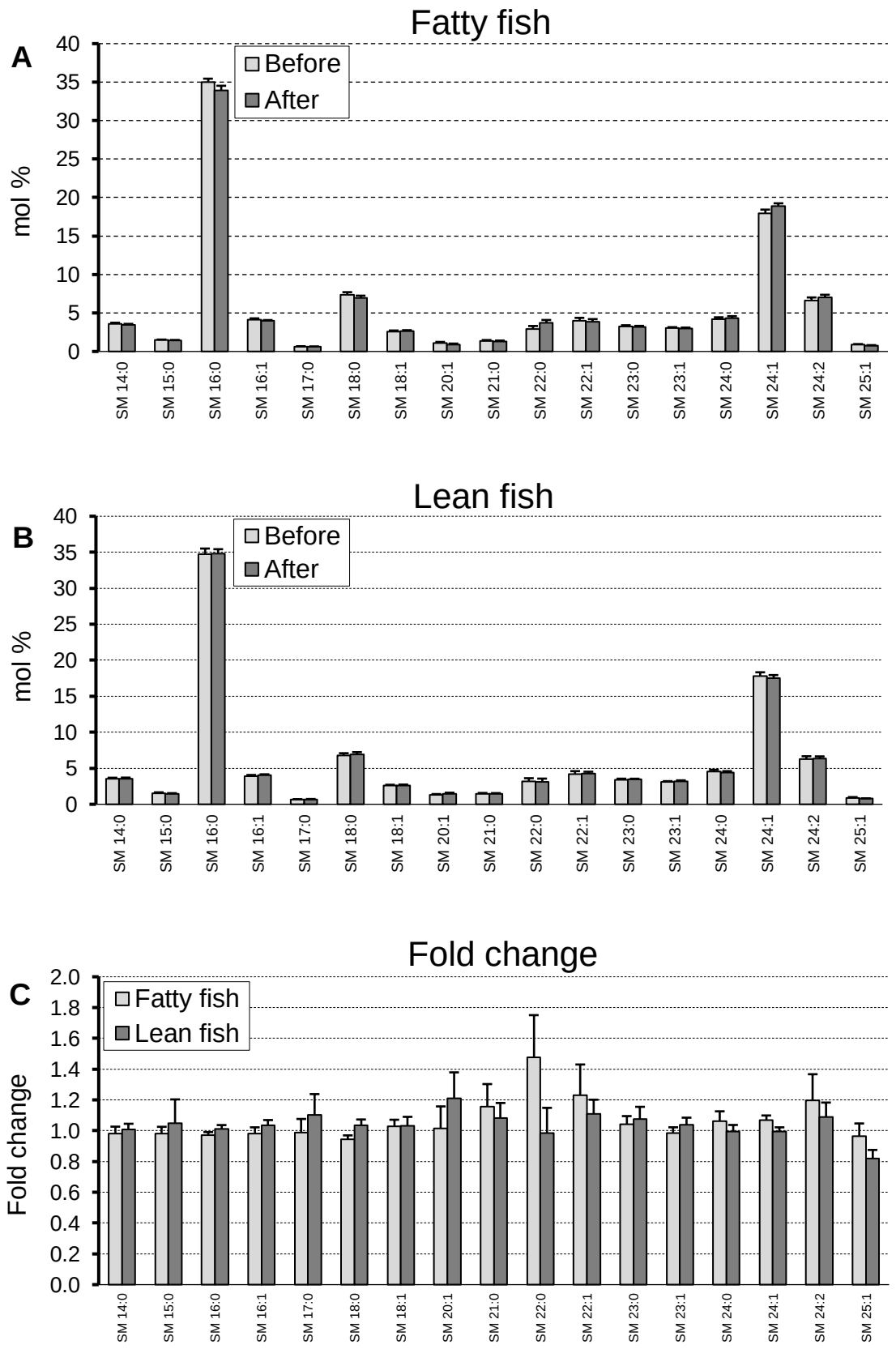


Figure 12: SM species lipid profile in fatty fish (A) and lean fish (B) groups before and after the experimental diet and (C) the fold change (mol% after 12 weeks/mol % at day 0) comparison of the two dietary groups. The acyl chain is abbreviated [chain length]: [number of double bonds].

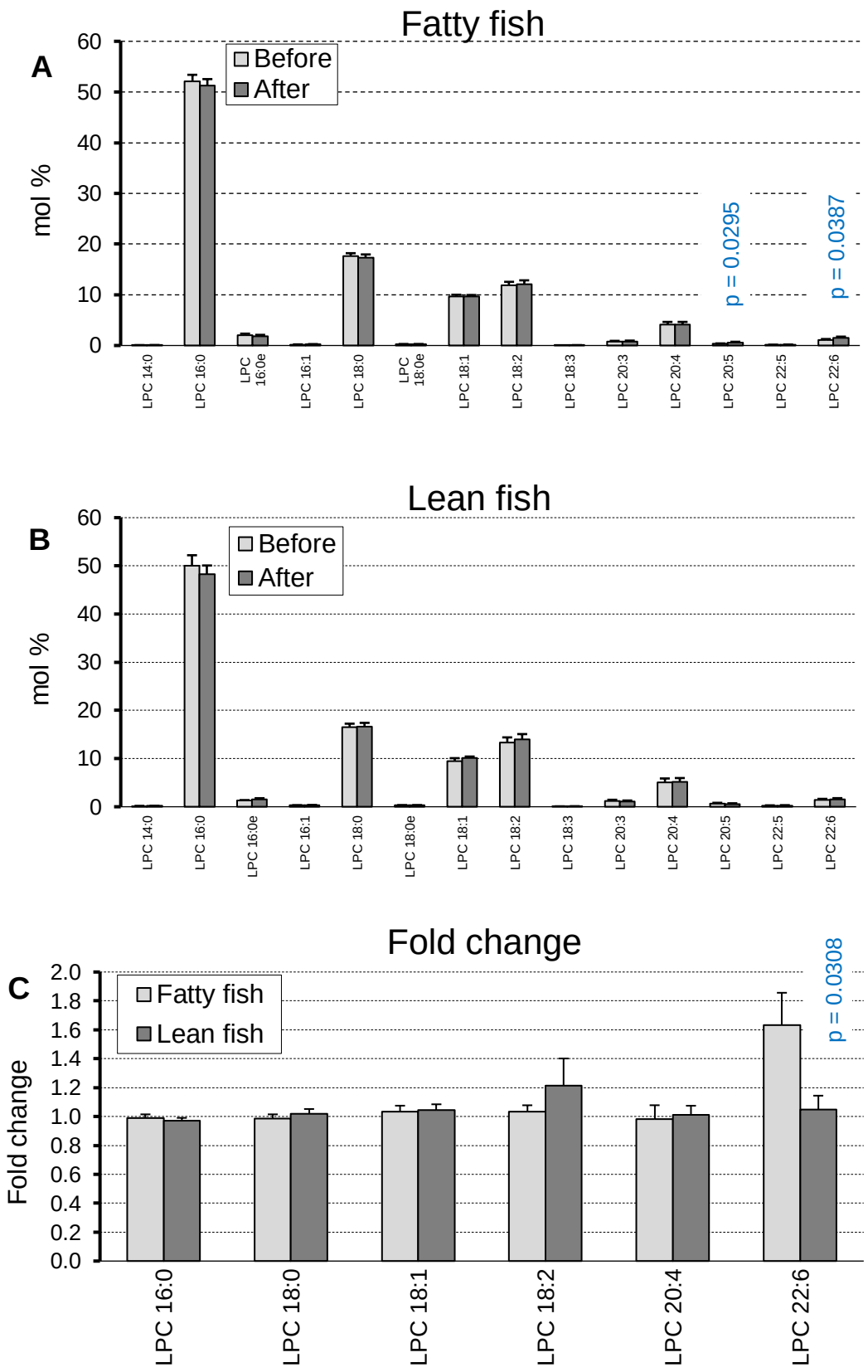


Figure 13: LPC species lipid profile in fatty fish (A) and lean fish (B) groups before and after the experimental diet and (C) the fold change (mol% after 12 weeks/mol % at day 0) comparison of the two dietary groups. The acyl chain is abbreviated [chain length]: [number of double bonds]. Statistical significance (p-value in blue) refers to Student´s paired t-test.

Of the core lipids of the LDL, the major lipid species found in both fatty fish and lean fish in CE were CE 18:2 (averages ranging between 59 and 62 mol% per total CE), CE 18:1 (averages ranging between 8 and 11 mol% per total CE), CE 20:4 (averages ranging between 9 and 10 mol% per total CE), CE 16:0 (averages ranging between 5 and 6 mol% per total CE), and CE 20:5 (averages ranging between 4 and 6 mol% per total CE) (Fig. 14). The CE species composition of the LDL particles showed significant changes due to the fatty fish diet (Fig. 14A). In Fig. 14A, significant increase in CE species CE 20:5 ($p = 0.0002$) was found. Along with the increase, significant decrease was found in species CE 16:0 ($p = 0.0439$), and CE 16:1 ($p = 0.0041$). Also, the fold change values for the CE showed changes in between the fatty and lean fish groups (Fig. 14C). Statistical significances were found with increase in species CE 20:5 ($p = 0.0004$), and CE 22:6 ($p = 0.0324$) in the fatty fish group.

Another core lipid class studied was TAG. The major lipid species found in both fatty fish and lean fish were TAG 52:2 (averages ranging between 18 and 19 mol% per total TAG), TAG 52:3 (averages ranging between 10 and 12 mol% per total TAG), TAG 50:2 (averages ranging between 8 and 9 mol% per total TAG), TAG 52:1 (averages ranging between 5 and 8 mol% per total TAG), TAG 50:1 (averages ranging between 6 mol% per total TAG), TAG 52:4 (averages ranging between 5 and 6 mol% per total TAG), TAG 54:2 (averages ranging between 5 and 6 mol% per total TAG), TAG 50:3 (averages ranging between 4 and 5 mol% per total TAG), and TAG 54:3 (averages ranging between 3 and 5 mol% per total TAG) (Fig. 15A, B). The TAG species composition of the LDL particles showed significant changes due to the fatty fish diet (Fig. 15A). As seen in Fig. 15A, significant decrease was found in minor species TAG 48:0 ($p = 0.0299$), and

major species TAG 50:2 ($p = 0.0456$). Besides, the fold change values for the TAG species was similar in both groups with minor changes (Fig. 15C).

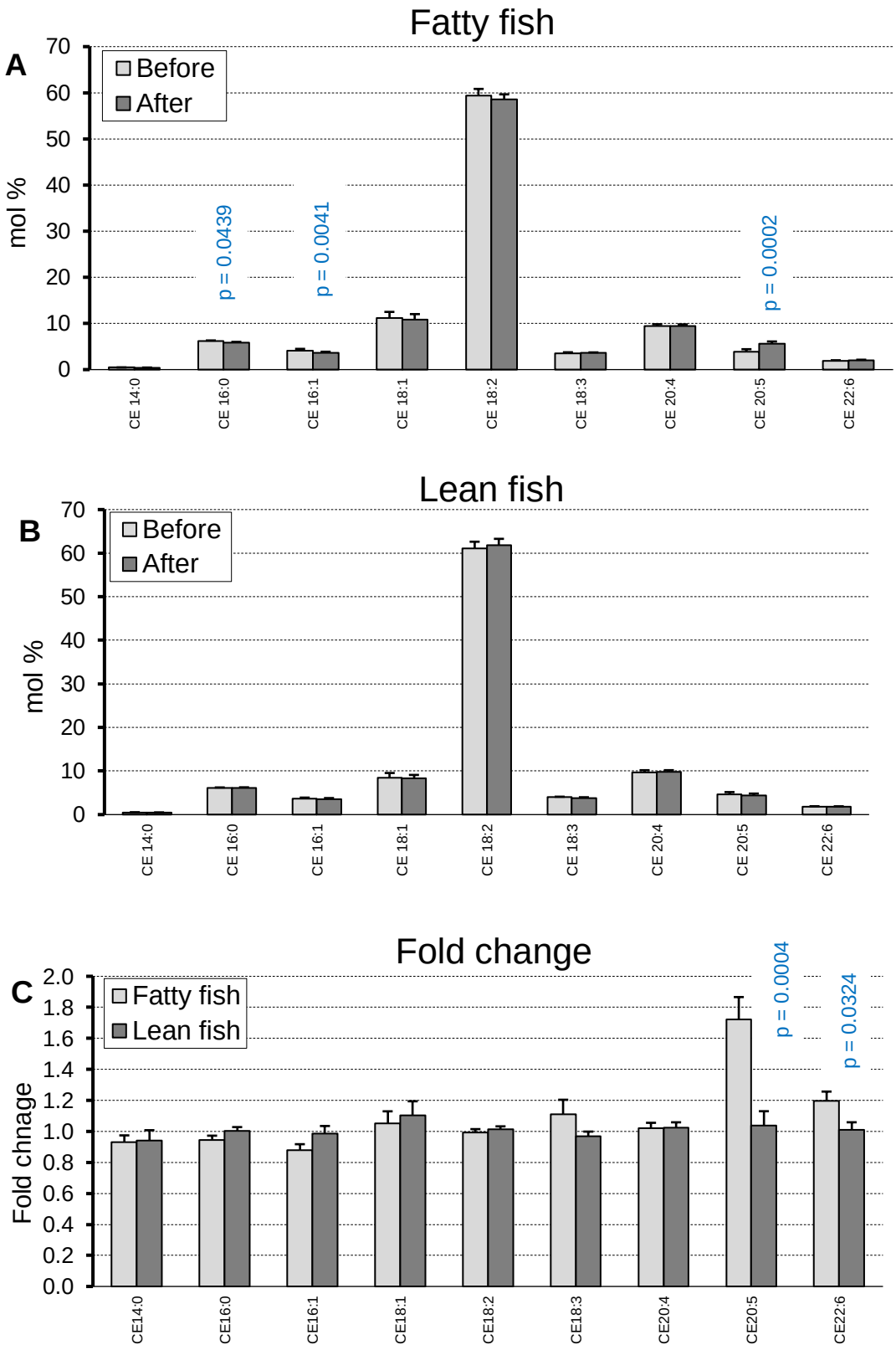


Figure 14: Cholesteryl ester species lipid profile in fatty fish (A) and lean fish (B) groups before and after the experimental diet and (C) the fold change (mol% after 12 weeks/mol % at day 0) comparison of the two dietary groups. The acyl chain is abbreviated [chain length]: [number of double bonds]. Statistical significance (p-value in blue) refers to Student´s paired t-test.

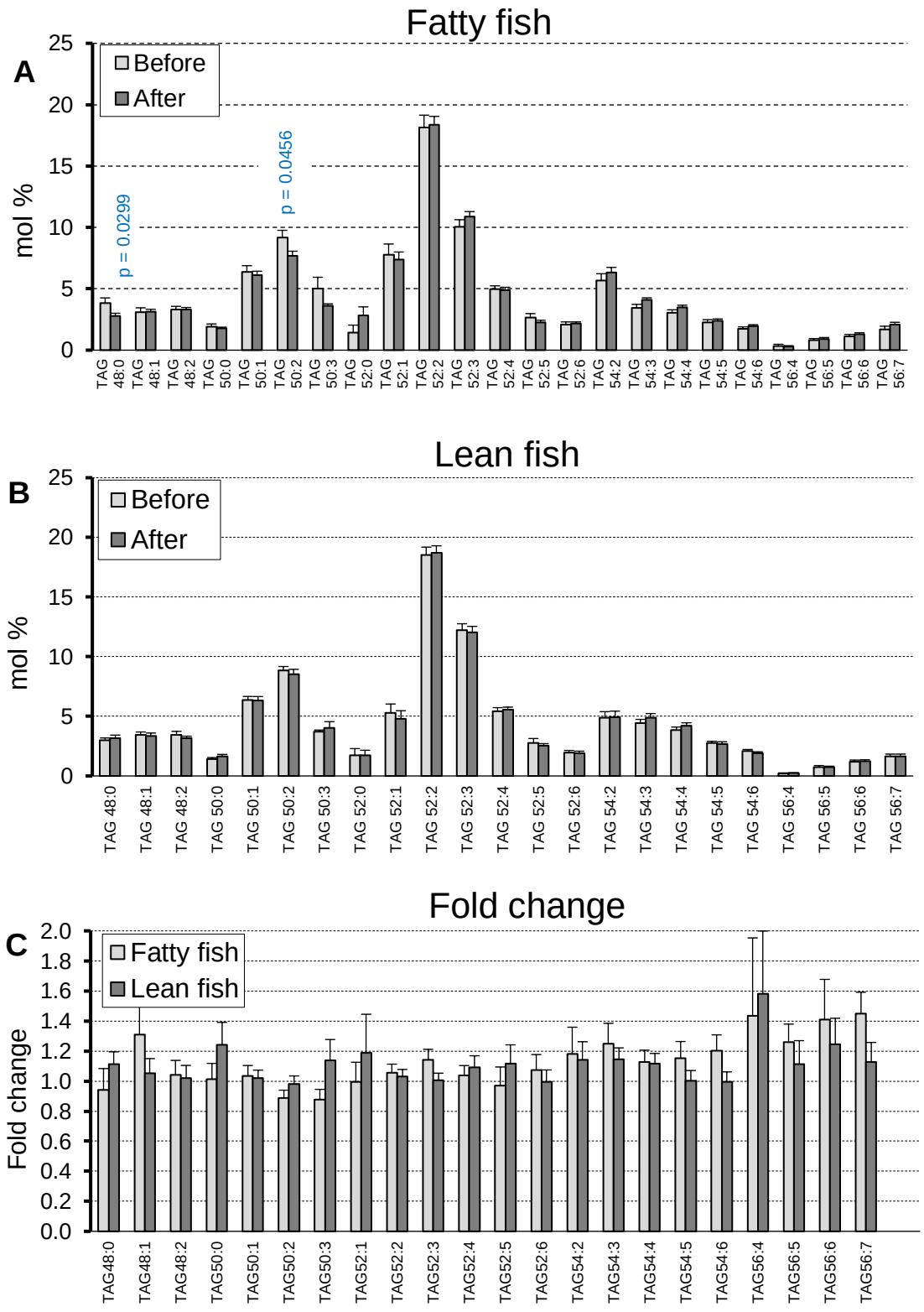


Figure 15: TAG species lipid profiles in fatty fish (A) and lean fish (B) groups before and after the experimental diet and (C) the fold change (mol% after 12 weeks/mol % at day 0) comparison of the two dietary groups. The acyl chain is abbreviated [chain length]: [number of double bonds]. Statistical significance (p-value in blue) refers to Student´s paired t-test.

5 Discussion

The objective of this study was to determine whether two types of fish diet: fatty fish and lean fish alter the lipid species profile of human LDL. The main lipid classes analysed in this study were LPC, PC, SM, TAG, and CE. Various previous studies have shown that EPA and DHA are the primary n-3 PUFAs of marine fish, freshwater fish, and consumption of these types of fish are beneficial for a healthy lifestyle (Kris-Etherton, Harris, and Appel 2002; Harris, Harris, and Harris 1997; Oelrich, Dewell, and Gardner 2011). EPA and DHA can be incorporated into different lipid classes. For example, they can be incorporated into membrane PLs and can be used as precursors for lipid mediators having anti-inflammatory and proresolving properties (Serhan, Chiang, and Van Dyke 2008). In this study, the lipid class profile did not change due to the fatty fish or the lean fish diet. Clear, although the not statistically significant change was found in TAG ($p = 0.0512$), with a decrease in the proportion of TAG by 19% after the fatty fish diet compared to the baseline values. The reduction in LDL-TAGs may reflect changes in TAG metabolism. Indeed, the consumption of PUFAs has been reported to associate with changes in the amounts of TAG in plasma. In a study conducted with normolipidemic individuals who were provided capsules of EPA and DHA, a decrease of 30% of total plasma TAGs was observed (Lindsey, Pronczuk, and Hayes 1992). In diet-based research conducted in 2002, in which 42 patients with type 2 diabetes were provided with either fish oil or corn oil supplements, 23% decrease in plasma TAG levels due to fish oil supplements was shown (Petersen et al. 2002), while the group of Suzukawa found a 24% decrease in TAG levels in healthy individuals (Suzukawa et al. 1995). Another diet-based study conducted in 2011 with 42 adults with hypertriglyceridemia, showed the effect of fish oil supplements with a 26% decrease in total serum TAG (Oelrich, Dewell, and Gardner 2011). The mean plasma TAG level of the participants in

the fatty fish group after the intervention was reported to be decreased, albeit not statistically significant (Oelrich, Dewell, and Gardner 2011).

There is a correlation between plasma TAG, chylomicron and VLDL levels as these lipoproteins are rich in TAGs (Oelrich, Dewell, and Gardner 2011). The concentration of TAG in lipoproteins depends on the balance between two factors: the rate of entry and the rate of removal of the TAGs from the plasma (Oelrich, Dewell, and Gardner 2011). A shift in the levels may be because of either of the two factors. The entry of TAG takes place at the ingestion of food, which is broken down and converted to free fatty acids and absorbed by intestinal cells and re-synthesized into TAG in chylomicrons and transported to lymphatics and further the bloodstream (Oelrich, Dewell, and Gardner 2011). Consumption of food increases the influx of TAG in blood. In this study, since the participants were on a fish-based diet, the amount of TAG transported to the blood might be reduced, which might affect the lipoprotein size. Therefore, it can be hypothesized that changes in the plasma TAG could affect the size of other lipoproteins.

The complete lipid class profile of LDL surface lipids does not show any changes in the profiles in either the fatty fish or the lean fish group. However, analysis of individual lipid species shows significant changes in the lipid species profile.

The PC species composition of the LDL particles showed a significant increase in PC species of PC 36:5, PC 38:6, PC 38:6e, and PC 40:6. PCs with five to seven double bonds most likely contain EPA (20:5n-3) or DHA (22:6n-3) acyl chains

even though the actual acyl chain composition was not determined in this study. Lankinen showed a substantial increase in PC 36:5, PC 38:7e and PC 40:7e in serum PLs after a 12-week healthy diet study containing fatty fish (Lankinen et al. 2011). Moreover, Ottestad observed a significant increase in plasma PC38:6 in a study investigating the effects of fish oil supplementation on plasma lipidomic profiles in healthy individuals (Ottestad et al. 2012). These results are in line with our observations from LDL lipidome, indicating that PCs containing EPA and DHA acyl chains accumulate into blood components with fatty fish of fish oil intake. The significant changes found in the lean fish group in this study were a decrease in the mol% of species of PC 32:1 and PC 40:5.

Previous research has shown that LPC, which is a proinflammatory lipid, plays a key role in various inflammatory diseases, including atherosclerosis, and diabetes (Schmitz and Ruebsaamen 2010; Lankinen et al. 2009). LPC alters functions of several cell-types such as endothelial cells, smooth muscle cells, and T-cells. Vasodilators such as nitric oxide are important factors that help endothelial cells to control smooth muscle cell tone. Evidence suggests LPC inhibit this activity of endothelial cells and causes vascular spasms (Takayuki Matsumoto 2007). Lecithin: cholesterol acyltransferase (LCAT) enzyme is a glycoprotein that is responsible for the formation of LPC and esterification of cholesteryl to CE by transferring a fatty acid from PC to cholesterol. LCAT is carried in HDL particles, but a small fraction also resides in LDL. Previous studies show that circulating LPC is associated with atherosclerosis (Orsó et al. 2015; Law et al. 2019). The LPC levels are increased in modified LDL, and oxidized LDL, but show an inverse relationship in plasma and serum in association with familial hyperlipidaemia and diabetes (Stübiger et al. 2012; Law et al. 2019). The topic of the effect of LCAT is still unclear because of many results showing increase LCAT activity with insulin resistance (Nass et al. 2018; Law et al. 2019), while

decreased LCAT activity in golden Syrian hamsters leads to atherosclerosis (Dong et al. 2018; Law et al. 2019). Few studies are showing the effect of diet on the LCAT activity; most have been conducted using rats (Romijn et al. 1998; Larking and Sutherland 1977). One such study conducted in humans in 2004 was in a French monk community where the diet cohort consisted of saturated fatty acids and divided into two experimental isocaloric groups; the results showed an increase in LCAT activity with decreasing dietary saturated fatty acids (Bérard et al. 2004).

An increase in two of the LPC species incorporating n-3 PUFAs (LPC 20:5 and LPC 22:6) was found in the fatty fish diet group. Ottestad had performed a diet-based study where healthy subjects showed an increase in the LPC species after intake of fish oil supplements (Ottestad et al. 2012). A study conducted by Lankinen and her group also showed a similar trend of increase in plasma LPC 20:5 and LPC 22:6 after a 12-week healthy diet containing fatty fish (Lankinen et al. 2009). Long-chain unsaturated plasma LPC 22:6, together with TAG 58:10 and PC 38:6, has been shown to associate with decreasing the risk of diabetes by the Framingham heart cohort study (Rhee et al. 2011; Ottestad et al. 2012). Per the results from previous research, the present study also showed a significant increase in plasma LPC 20:5 and LPC 22:6 due to the fatty fish diet. Based on the results, it can be assumed that fatty fish diet causes remodelling of the LDL lipidome profile in favour of more long-chained unsaturated fatty acids such as EPA and DHA species of LPC which have been shown to reduce inflammation and decrease the rate of heart diseases.

SM is mainly secreted into circulation through VLDL and chylomicrons; major SM species present in these two lipoproteins are SM 16:0 and SM 18:0 (Salpeter and Zilversmit 1968; Nilsson and Duan 2006). The SM which is present in LDL and HDL are derived from the lipoproteins mentioned above after VLDL and chylomicrons are transported to peripheral tissues to hydrolyse. Therefore, finding the same species in abundance in LDL particles can be expected (Nilsson and Duan 2006). Major SM species found in LDL particles were SM 16:0, SM 18:0, SM 24:1, and SM 24:2. No significant changes were found in the SM species profile of either the fatty fish or the lean fish group. The result of this study, along with another previous study conducted by Myher, shows that plasma SM composition is relatively stable (Myher et al. 1981). In another study, increase in three SM lipid species in plasma [SM(d18:0/20:0), SM(d18:0/22:6), SM(d18:0/26:2)] was observed in fish oil supplement group compared to high oleic sunflower oil group in a diet-based study conducted in healthy subjects (Ottestad et al. 2012). In that study, Ottestad showed that fish oil supplementations had no association with changes in plasma ceramides (Ottestad et al. 2012). In contrast, Lankinen and colleagues showed a decrease in total ceramide concentrations due to fatty fish consumption (Lankinen et al. 2009). Previous research shows the association of plasma ceramides with CVD and specifically linkage of high amounts of SM 24:0 to less atherogenic lipoprotein particles, but not less LDL in healthy subjects (Pfeiffer et al. 2001; De Mello et al. 2009; Hammad et al. 2010; Ottestad et al. 2012). As discussed previously, the main reason for the development of atherosclerosis is increased levels of LDL cholesterol as it leads to aggregation of the LDL particles in the arterial intima and stepwise modification of the LDL by various enzymes. A recent paper using hrSMase (human recombinant secretory sphingomyelinase) showed that an increased proportion of SM in LDL particles is associated with increased aggregation susceptibility of LDL particles and is associated with increased cardiovascular death (Ruuth et al. 2018). No difference in the aggregation

susceptibility of the LDL particles after consumption of fatty or lean fish was found (Manninen et al. 2019). In concordance with this finding, lipidomic analysis of the LDL samples performed here did not indicate any differences in the SM/PC ratio in the LDL particles.

A study conducted by Lindsey and her group showed an increase in CE 20:5 accompanied by a decrease in CE 18:2 with the uptake of fish oil (Lindsey, Pronczuk, and Hayes 1992). Two studies conducted with fish oil and fish diet respectively showed an increase in cholesterol concentration in small LDL particles with no change in the LDL size (Patti et al, 1999) and a non-significant increase in the concentration of small LDL predicting the probability of conversion of VLDL to LDL, i.e., lipolysis (Li et al. 2004). It was hypothesised that even though fish oil and fish diet elevated the levels of cholesterol in small LDL, making them dense, the effect was not sufficient enough to change the LDL size (Patti et al, 1999). To some extent in the present diet study, fatty fish diet altered LDL core composition by inducing a 1.7-fold increase in the proportion of CE 20:5 and 1.2-fold increase in the proportion of CE 20:6, which was accompanied by a decrease in CE 16:0 and CE 16:1, showing that fatty fish diet can change the composition of LDL particles without influencing the particle size.

Statistically significant changes were found in TAG species profiles in the fatty fish group in this study. A minor TAG species TAG 48:0 and a major species TAG 50:2 showed a significant decrease. Fish oil supplements are shown to lower plasma TAG levels (Oelrich, Dewell, and Gardner 2011). One of the main results of Ottestad showed in their diet study that increases in plasma long-chain TAG

56:9 and TAG 58:10 due to intake of fish oil supplements are associated with reducing the risk of diabetes (Ottestad et al. 2012). Fish oil supplements also are shown to inhibit the synthesis of VLDL, therefore, reducing the number of plasma TAGs (Nestel et al. 1984; Oelrich, Dewell, and Gardner 2011). A decrease in TAG 48:1, TAG 48:2, and TAG 50:2 were seen with n-3 fish oil supplements in a 24-week weight loss in obese women (McCombie et al. 2009). Similarly, a decrease in TAG 48:1 and TAG 48:2 which was observed in a knock-out mouse model of PPARg2$^{-/-}$ Lepob/Lepob (Peroxisome proliferator-activated receptor-gamma), which is essential for the development of adipose tissues (Medina-Gomez et al. 2007) showing a selective modification of TAGs in obesity and type-2 diabetes (McCombie et al. 2009). Compared to these studies, the present study showed a decrease in only TAG 50:2, which can be hypothesised to play role in lowering the risk of obesity, for example. Previous dietary interventions show a significant increase in long-chain polyunsaturated TAGs with fish consumption for eight weeks and lean fish but not fatty fish (Lankinen et al. 2009; Ottestad et al. 2012). Whereas no significant changes were found in the TAG species profile of the lean fish group.

A three-layer model (consisting of outer surface layer, interfacial layer, and the core) for LDL particle in terms of the proper orientation of the PL at the surface to the completely random orientation of the core lipids has been proposed (Saito et al. 1996; Deckelbaum, Shipley, and Small 1977; Hevonoja et al. 2000). According to this model, the lipid classes in LDL particles are not homogeneously distributed, and the dynamic state of the particles contributes to the change in the structure of the particles along with a change in the composition of the lipids (Hevonoja et al. 2000). It might be one reason for the alterations in some of the surface as well as core lipids of the LDL particles. Rhee et al. showed that LC-MS based lipid profiling could be used for the identification of the relationship

between the lipid acyl chain and the double bond content and the risk of disease (Rhee et al. 2011). Selective modifications such as an increase in the TAG and PL species having long-chain highly unsaturated fatty acids of high carbon number lay behind the potential positive effect of fish oil supplements (Ottestad et al. 2012).

6 Conclusion

The lipid class profile did not change due to the two fish type diets. The lipid classes that mainly contributed to the changes in the LDL lipidome profiles in this study were PC, LPC, CE, and TAG. The consumption of fatty fish diet increased the levels of lipid species of PC, LPC, and CE containing EPA and DHA acyl chains, while decreasing levels of two TAG species. Elevated levels of PC 38:6 and LPC 22:6 were shown to help in decreasing the risk of diabetes by the Framingham heart cohort study (Rhee et al, 2011; Ottestad et al. 2012). Elevated levels of the same species were also observed in the present study indicating to some degree that fish diet is one of the essential facets in the reduction of risk of diabetes. Although the composition of core CEs increased in certain species, the overall CE composition remained unchanged. The present study did not show any changes in the SM species, which can indicate that fish diet specifically n-3 PUFAs from fish, might not have any effect in altering the composition of SM in the lipidome. There are various pharmacological agents such as niacin and fenofibrate, in the market already used for the reduction of TAG (Ford et al, 2002; Oelrich, Dewell, and Gardner 2011). This study, along with others, shows that diet containing fatty fish can decrease short-chain TAGs of plasma LDL, and provide another potential approach that could be beneficial than consuming medicines. Lean fish induced minor changes in the lipid composition of LDL particles. In this study, a moderate sample size was studied,

which included middle-aged volunteers with impaired fasting glucose. The results acquired from this study could be limited to this age group. Further investigations of the effect of fish diet might be imperative with larger sample size and different age groups. In conclusion, fatty fish diet alters the plasma lipidome profile and the changes induced to both the surface and the core lipid composition of the LDL particles may affect the physical properties of the LDL particles and their size.

7 Acknowledgement

This thesis was a work in progress for quite some time. But I am glad to see it finally being done. The thesis was not a one-woman show. I would like to sincerely thank both my supervisors Reijo Käkelä and Katariina Öörni for being so patient with me and helping me understand some basic concepts and not giving up on me. I would also like to thank Minna Holopainen, Sanna Sihvo, Hanna Ruhanen, Feven Tigistu-Sahle and Malin Tverin for helping me with explaining the data and creating such a good atmosphere in the workplace. It was a real pleasure to work with you all. Last shoutout goes to my friends in Helsinki and my parents Mr and Mrs Bhalke for always being there for me no matter what!

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